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**Succession of Nematode Fauna and
Fungal Flora in Pine Trees after Infection
with the Pinewood Nematode**

Rina Sriwati

2007

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GENERAL INTRODUCTION

Pine wilt disease, caused by the pinewood nematode (PWN), *Bursaphelenchus xylophilus* Steiner and Buhrer, is a major threat to pine forests in Japan and causes serious damage to the most common native pines, *Pinus densiflora* Sieb. and Zucc., *P. thunbergii* Parl. and *P. luchuensis* Mayr (Mamiya, 1988). Since the first occurrence in Nagasaki, Kyushu, in 1905 (Yano, 1913), this devastating epidemic has rapidly spread throughout Japan, except for the two northernmost prefectures, Aomori and Hokkaido, out of 47 prefectures. In 2000, infested areas were estimated to be 27.6% of Japanese pine forest (2.1 million ha) (Mamiya, 2004). This disease, presumably originating in North America (de Guiran and Bruguir, 1989), has spread so rapid also in other Asian countries (Yang, 2004). Moreover, recently it has been found also in Portugal (Mota *et al.*, 1999).

The PWN is transmitted mainly by the Japanese pine sawyer, *Monochamus alternatus* Hope, from wilt-killed to other healthy pine trees (Mamiya and Enda, 1972; Morimoto and Iwasaki, 1972). The adult beetles of *M. alternatus* carrying a great number of PWNs in their trachea emerge from PWN-killed pine trees in early summer. Newly-emerging adults fly to healthy trees and feed on the bark of young twigs for maturation of reproductive organ. At that time, PWNs on the vector beetles are transmitted to healthy trees and invade them through the feeding wounds made by the beetles. A small number of PWNs disperses widely in the infected trees and causes the cessation of oleoresin flow. Thereafter PWNs propagate dramatically and the trees show wilting symptoms, releasing volatiles such as ethanol, terpenes and so on (reviewed by Kishi, 1995). Mature beetles are attracted to these wilting trees and lay their eggs on

them. The eggs hatch within a week and the larvae feed on the inner bark and outermost sapwood, then, bore into the sapwood to form pupal chambers in autumn. The number of PWNs reaches its maximum from autumn to winter, then, decreases gradually (Fukushige and Futai, 1987; Mamiya *et al.*, 1973). Pupal chamber of *M. alternatus* beetles is one of the most important places for PWN. Maehara and Futai (2002) reported that numerous PWNs aggregated around pupal chambers of *M. alternatus* in wilt-killed pine trees and that the beetles emerging in the subsequent year harbored many nematodes on their bodies.

PWNs, which are transmitted to healthy trees, feed on the parenchyma cells of the trees and on fungi such as *Pestalotia* spp. and *Rhizosphaera* spp., which sparsely distribute in living trees. When host tree is diseased, food sources of PWNs must be replaced with various wood-inhabiting fungi such as blue-stain fungi (Kobayashi, 1975; Kobayashi *et al.*, 1975; Iwahori and Futai, 1990; Fukushige, 1991), though such fungi as *Trichoderma* spp. also inhabiting in dead pines are unsuitable for PWN propagation (Kobayashi *et al.*, 1975; Fukushige, 1991; Maehara and Futai, 1997).

Under field conditions, dying trees in general are rapidly invaded by various wood-decaying microorganisms (Shigo, 1967) and intense competition among such microorganisms brings about a succession of microbial flora and fauna. Abiotic environmental conditions, especially temperature, moisture and substrates and/or biotic factors greatly affect the succession of organisms.

A previous study clarified the seasonal changes in the numbers of PWNs and other free-living nematodes, and examined their interrelationships (Fukushige and Futai, 1987). However, that study did not distinguish the species comprising the 'free-living nematodes' and so the interaction between the PWN and each species of free-living

nematode has remained unclear. Some researchers have clarified changes in fungal flora inhabiting dead pine trees (Kobayashi *et al.*, 1974, 1975; Maehara and Futai, 2000; Wang *et al.*, 2005). Among the fungal species isolated from wilt-killed pine trees, some have been known to be suitable food source for the PWN, e.g., *Ceratocystis* sp., *Diplodia* sp. and *Pestalotia* sp., while others to be unsuitable, e.g., *Trichoderma* spp., *Verticillium* sp., *Cephalosporium* sp. (Kobayashi *et al.*, 1974, 1975; Fukushige, 1991). Under laboratory conditions, Maehara and Futai (1996, 1997) demonstrated that each fungal species that proliferated around the pupal chamber of *M. alternatus*, affected not only PWN multiplication but also the number of PWN carried by the vector beetle. These findings clearly indicate that fungal flora in a dead pine tree might be one of the most determinant biotic factors for the multiplication and distribution of PWN inside the tree.

This study focused on the changes in nematode fauna and in microbial flora in pine trees after infection with PWN, and thereby clarified their relationship together with the population dynamics of PWN.

In the first chapter, I investigated the nematode fauna in the stems of pine trees which were harvested every other month after infection with the PWN to clarify their seasonal changes, and examined the interrelations between the PWN and the other species comprising the nematode fauna. I also examined the effect of pupal chambers bored by *M. alternatus* on the population density of nematode species inhabiting in the dead pine trees. In the second chapter, I described the bionomics and general characteristics of 15 nematode species that constituted nematode fauna in pine trees killed by PWN. In order to clarify the relationship between PWN and fungi cohabitating in dead pine trees, I also investigated seasonal change in the fungal flora in the trees and

analyzed the effect of fungal flora on the distribution and population density of PWNs at the microhabitat level (Chapter III). In Chapter IV, I examined PWN propagation on 18 fungi isolated from dead or dying pine trees, under laboratory conditions, and compared the results with those obtained from field grown trees.

CHAPTER I

Seasonal changes of nematode fauna in pine trees killed by the pinewood nematode, *Bursaphelenchus xylophilus*.

Introduction

In the pine trees that were killed by pinewood nematodes (PWN), blue stain fungi proliferating on the walls of pupal chamber (PC) of sawyer beetle, *Monochamus alternatus* serve as a food source for PWNs, thereby PWNs propagate and aggregate around the chambers. When *M. alternatus* eclose, numerous PWNs transfer into the tracheal tubes of the beetle. And then emerging beetle carries many nematodes to inoculate them into healthy trees during maturation feeding.

Thus, the beetles that emerged from the PCs acquire many PWNs both externally and internally (Maehara and Futai, 1996; Maehara and Futai, 2001; Maehara *et al.*, 2005).

When trees become diseased, the physical conditions within the trees, such as water content and temperature, change dramatically (Shigo, 1967). This invokes corresponding changes in microbial flora in the trees, indicating that the nematode fauna should also change promptly.

An earlier study clarified the seasonal changes in the numbers of PWNs and other free-living nematodes, and their interrelationships (Fukushige and Futai, 1987).

However, that study did not show the species composition of the ‘free-living nematodes’ and so the interaction between the PWN and each species of free-living nematodes remained unclear.

A wide variety of nematode species was associated with dead *Pinus koraiensis* Sieb. & Zucc., trees in Primorye, Russia (Kruglik, 2003), and most of them were mycophagous or saprophagous nematodes. Among them nematodes belonging to the order Aphelenchida were quite abundant in dead wood of the Russian trees.

In the present chapter, I investigated the nematode fauna in the stems of pine trees bimonthly after infection with the PWN to: (i) clarify their seasonal changes and the interrelations between the PWN and the other species comprising the nematode fauna, and (ii) examine the effect of PCs of *M. alternatus* on the population density of the nematode species inhabiting dead pine trees.

Materials and Methods

PWN INOCULATION

A virulent isolate (S10) of the PWN, was cultured on the mycelium of *Botrytis cinerea* Pers. grown on barley grain medium (unhulled barley grain 10 ml; tap water 10 ml, autoclaved at 121°C for 20 min) for one month. The Baermann funnel method was used to extract the nematodes from the fungal colonies, then the number of nematodes in the suspension was adjusted to 10,000 nematodes / ml. Inoculation of the trees was done on 10 June, 2004, at the Kamigamo Experimental Station of the Field Science Education and Research Center, Kyoto University where located on a slope (average inclination: 29.7°) facing east-north-east (35°04'-N, 137°31'-E, 140 m above sea level),

Kyoto, Japan. Fifteen-year-old Japanese black pines, *P. thunbergii* (average diameter at breast height was 5.3 cm and standard deviation was 3.5 cm) were inoculated as follows. The aliquot nematode suspension of 0.5 ml was injected into a hole drilled in the bark at about 2.5 m above ground line. Cotton balls were put in the hole and another 0.5 ml aliquot of the nematode suspension was injected, i.e. 10,000 PWNs were injected per tree. The inoculation wound was sealed with Parafilm® (Pechiney Plastic Packaging, Inc. Chicago, IL, USA). Eighteen pine trees whose diameter at breast height ranged from 3.6 to 7.7 cm were inoculated with PWNs, and the another three trees with diameter from 5.3 to 5.7 cm were injected with the same volume of distilled water (the control treatment). The controls were needed to determine if the trees had been infected before inoculation and to compare their results with those of the PWN-inoculated trees.

INTRODUCTION OF *M. ALTERNATUS* LARVAE INTO PINE STEMS

The *M. alternatus* adults emerging from logs of dead, Japanese black pine located at the Arid Land Research Center, Tottori University, in the Tottori sand dune, Tottori, Japan (35°32'N, 134°13'E) where natural pine forests had been suffered from the pine wilt, were trapped. A pair of beetles was reared in a small cage and fed young pine twigs for food and oviposition. After 4 days, the eggs laid were collected from the twigs, dipped in 70% ethanol for 10 sec and in 0.05% benzethonium chloride for 5 min, and then rinsed three times in sterile, distilled water (Kosaka and Ogura, 1990; Kosaka and Enda, 1991). The eggs were then placed in microplate wells, each containing 500 µl of 1/10 PDA medium, and kept under aseptic conditions until they hatched.

On 6 July, 2004, when the inoculated pine trees had ceased resin exudation, the

stem of each tree was drilled at 8 points (each 25 cm apart) at a height of 75 to 250-cm above ground and using a paintbrush one 1st stage larva of *M. alternatus* was introduced into each hole.

WOOD SAMPLING

On 10 August, 13 October and 16 December, 2004, and 9 February, 10 April and 10 June, 2005, three dead trees were felled and a 10-cm-long wood block including one of the points where a larva was introduced, and where a PC had been made, was arbitrarily collected from each tree. Each of the wood blocks was sliced into 8, 1-cm thick discs, and 2 x 2 cm lattices were drawn on the cut surfaces. Then the discs were photocopied to record the position of PCs and tunnels of *M. alternatus*. Then each of the discs was cut into 2 x 2 cm squares along the line of the lattice, and each piece was split into two halves (2 x 1 x 1 cm). One half was used for isolating the nematodes and the other half for fungal isolation.

Each of the half pieces was sliced into small chips with pruning shears and placed in a Baermann funnel for 40 hours to extract nematodes. After extraction, the chips were dried overnight at 60°C to determine their dry weight. The nematodes extracted were killed in water bath at 70 – 80°C for 2 min and fixed in TAF (triethanolamine 2 ml; formalin 7 ml; distilled water 91 ml). Using a stereomicroscope and nematode counting slide the number of the nematodes of each species was recorded.

MORPHOLOGICAL IDENTIFICATION OF THE NEMATODES

The nematodes collected were processed by a quick method, consisting of

fixation with warm lactophenol and preservation in glycerine (Siddiqi, 1964). The specimens were then mounted on slides and identified using a light microscope. Several species characters were used for identification according to Maggenti's system (Maggenti, 1991).

DATA ANALYSIS

Nematode numbers were calculated as numbers of nematodes per gram of wood sample (dry weight basis). For the two most prevalent nematode species, *Diplogasterida* sp. 1 and *B. xylophilus*, a regression analysis was done after log-transformation of their numbers. Also, the water content of the samples and log-transformed numbers of *B. xylophilus* was regressed. Samples that contained no *B. xylophilus* were excluded from the analysis.

The ratio of nematode numbers in the wood samples with the PC and/or tunnel fractions, to those in the wood samples without PC or tunnel fractions was calculated for each disc, and thereby the degree of aggregation of each nematode species to the PCs and tunnels of *M. alternatus* was evaluated.

Results

SEASONAL CHANGES IN NEMATODE FAUNA

Seasonal changes in nematode fauna and the population density of each nematode species including the PWNs are shown in Fig. 1. During the experimental period, 15 nematode species were detected including the (i) five mycophagous species: *B. xylophilus* (PWN), *Bursaphelenchus* n. sp., and *B. sinensis*, Tylenchida sp. 1,

Tylenchida sp. 2; (ii) nine saprophagous species: Diplogasterida sp. 1, Diplogasterida sp. 2, Diplogasterida sp. 3, Diplogasterida sp. 4, Monhisterida sp. 1, Monhisterida sp. 2, Rhabditida sp. 1, Rhabditida sp. 2, Plectidae sp.; and (iii) one predacious species Mononchida sp. (for detailed descriptions and pictures, see Chapter II). Over all seasons, *B. xylophilus* was the most abundant species, followed by Diplogasterida sp. 1.

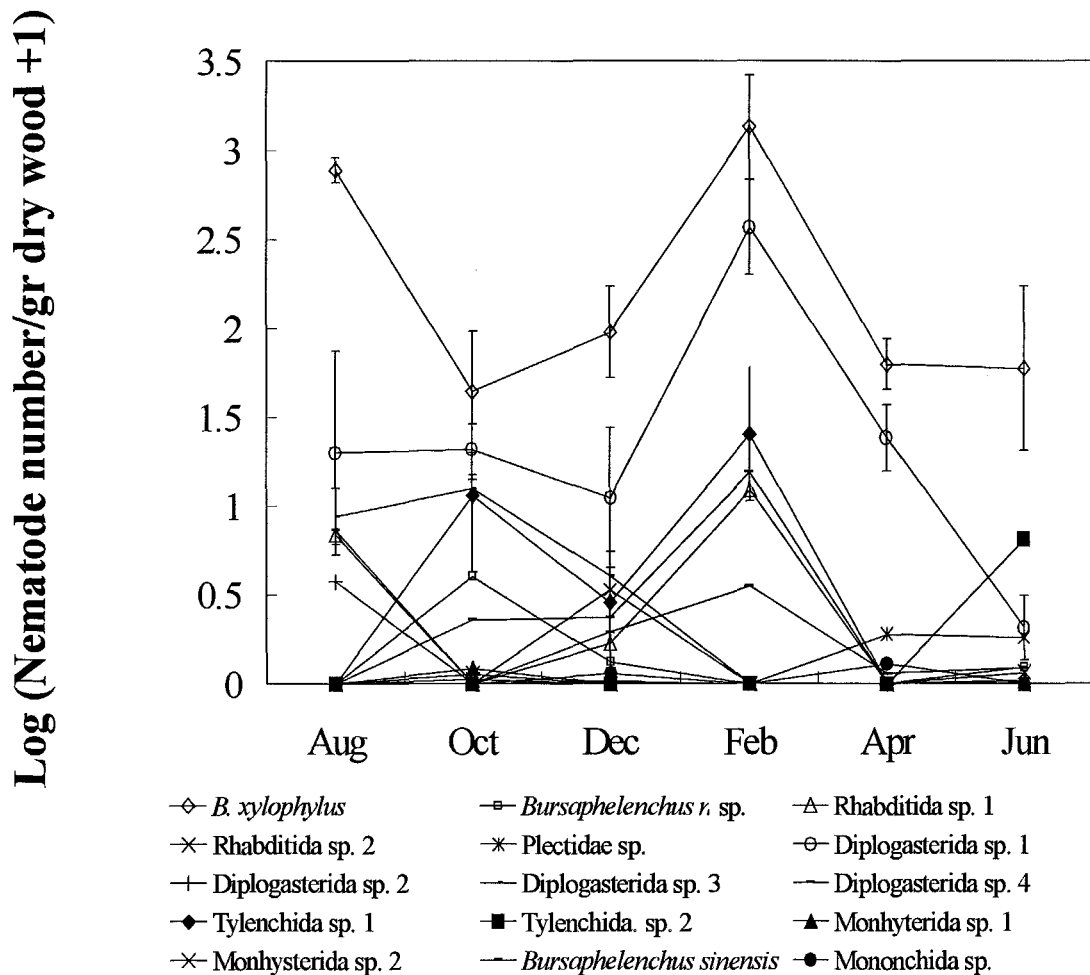


Fig. 1. Seasonal changes of the nematode population of each species on dead pine trees. Each bar indicates standard error (n=3).

INTERRELATION BETWEEN *B. xylophilus* AND DIPLOGASTERIDA SP. 1 IN DEAD PINE TREES AND ITS SEASONAL CHANGE

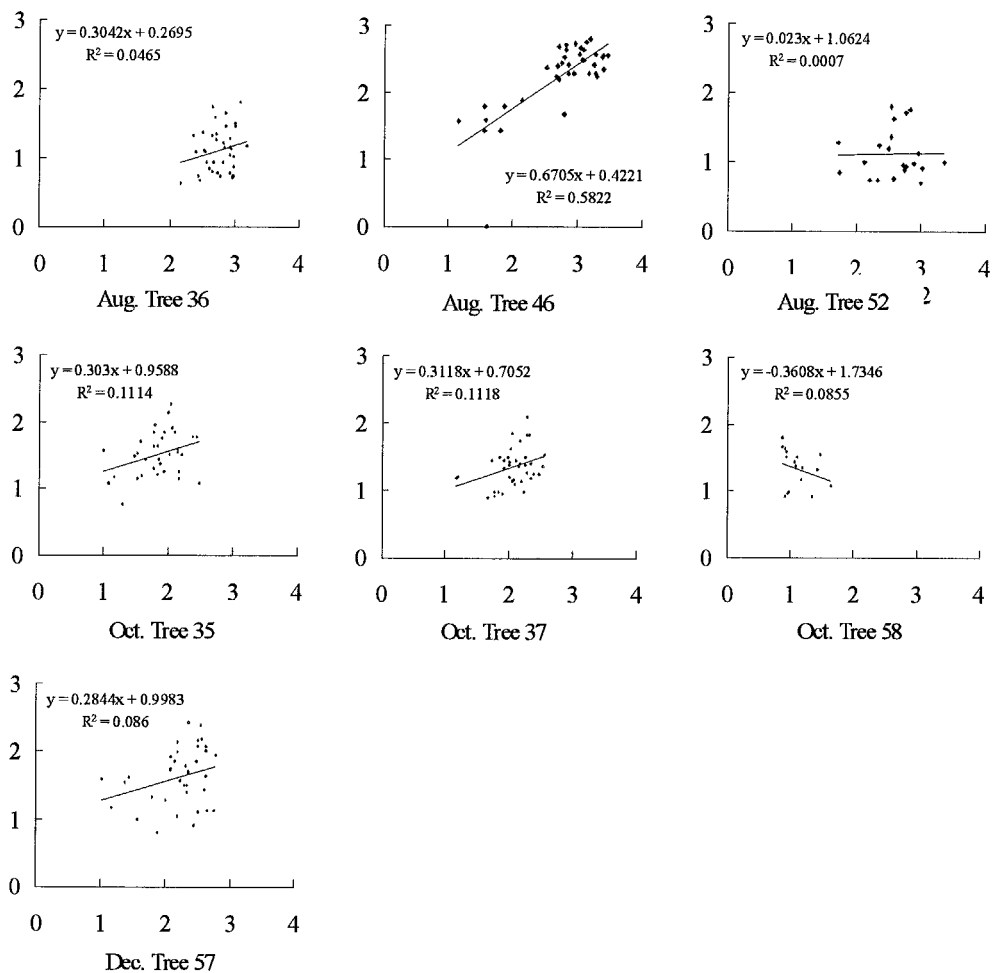
Figure 1 shows that the population density of *Diplogasterida* sp. 1 changed synchronously with that of *B. xylophilus* throughout the experimental period. However, the correlation between those two populations in the wood samples varied from tree to tree (Fig. 2). Even in the same sampling season, except for February, no significant correlation was indicated between populations of the two nematode species. In some trees during December and June neither *B. xylophilus* nor *Diplogasterida* sp. 1 were found; consequently no correlation analyses could be made.

EFFECT OF *M. ALTERNATUS* PUPAL CHAMBERS ON THE NUMBER OF NEMATODES

Figure 3 shows the ratio between the numbers of nematodes in the wood samples with PCs and/or tunnel fractions to those in the wood samples without them.

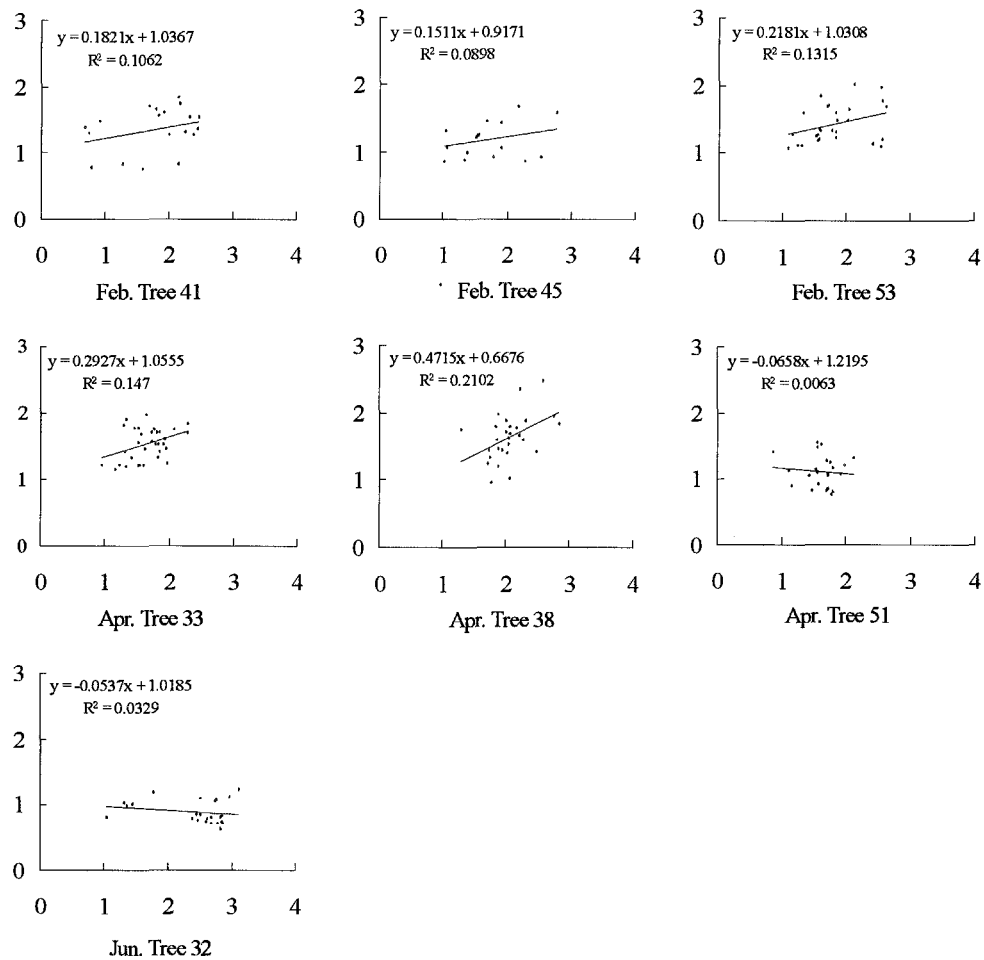
Beetle tunnels were first found on trees sampled in August and PCs first appeared in October. Both of the dominant species, *B. xylophilus* and *Diplogasterida* sp. 1, were more abundant in the wood samples with PCs and/or tunnel fractions than in those lacking such structures. Thus, these two species preferably aggregated to PCs and tunnels. The other nematode species did not show any preferable aggregations to PCs or tunnels and at some sampling dates they were not detected.

Log (Number of Diplogasterida sp. 1 /gr dry wood +1)



Log (Number of *B. xylophilus* /gr dry wood +1)

Log (Number of *Diplogasterida* sp. 1 /gr dry wood +1)



Log (Number of *B. xylophilus* /gr dry wood +1)

Fig. 2. Relationship between the number of *B. xylophilus* and that of *Diplogasterida* sp. 1 on dead pine trees.

RELATIONSHIP BETWEEN THE NUMBER OF *B. XYLOPHILUS* AND THE WATER CONTENT OF DEAD PINE TREES.

The relationship between the water content of wood samples and the population density of PWNs varied from tree to tree (Fig. 4). In December positive correlation was observed between these two parameters, though no consistent correlation was observed in other sampling times.

Discussion

Previous studies on the seasonal change in nematode fauna in dead pine trees focused on the number of the PWNs and ‘free-living’ nematodes, however, the latter ones were not classified into species, but instead just recorded as ‘free-living nematodes’ (Futai *et al.*, 1986; Fukushige and Futai, 1987).

In the present study, 15 species of nematodes were identified, including mycophagous species, such as two species of Tylenchida and two species of *Bursaphelenchus*, and nine saprophagous species. Two species of Tylenchida were considered to be mycophagous because they reproduced on fungus mycelium which grew from the wood on PDA. Interestingly, males of the undescribed Aphelenchida species had a bursa, a distinctive characteristic of the genus *Bursaphelenchus*, however, females of the same species had an indistinct anus or lacked one, which is a characteristic of nematodes in the genera *Ektaphelenchus* and *Cryptaphelenchus*. Braasch (2004) described a *Bursaphelenchus* species with characteristics like those of Ektaphelenchidae species collected in China. The *Bursaphelenchus* species described by

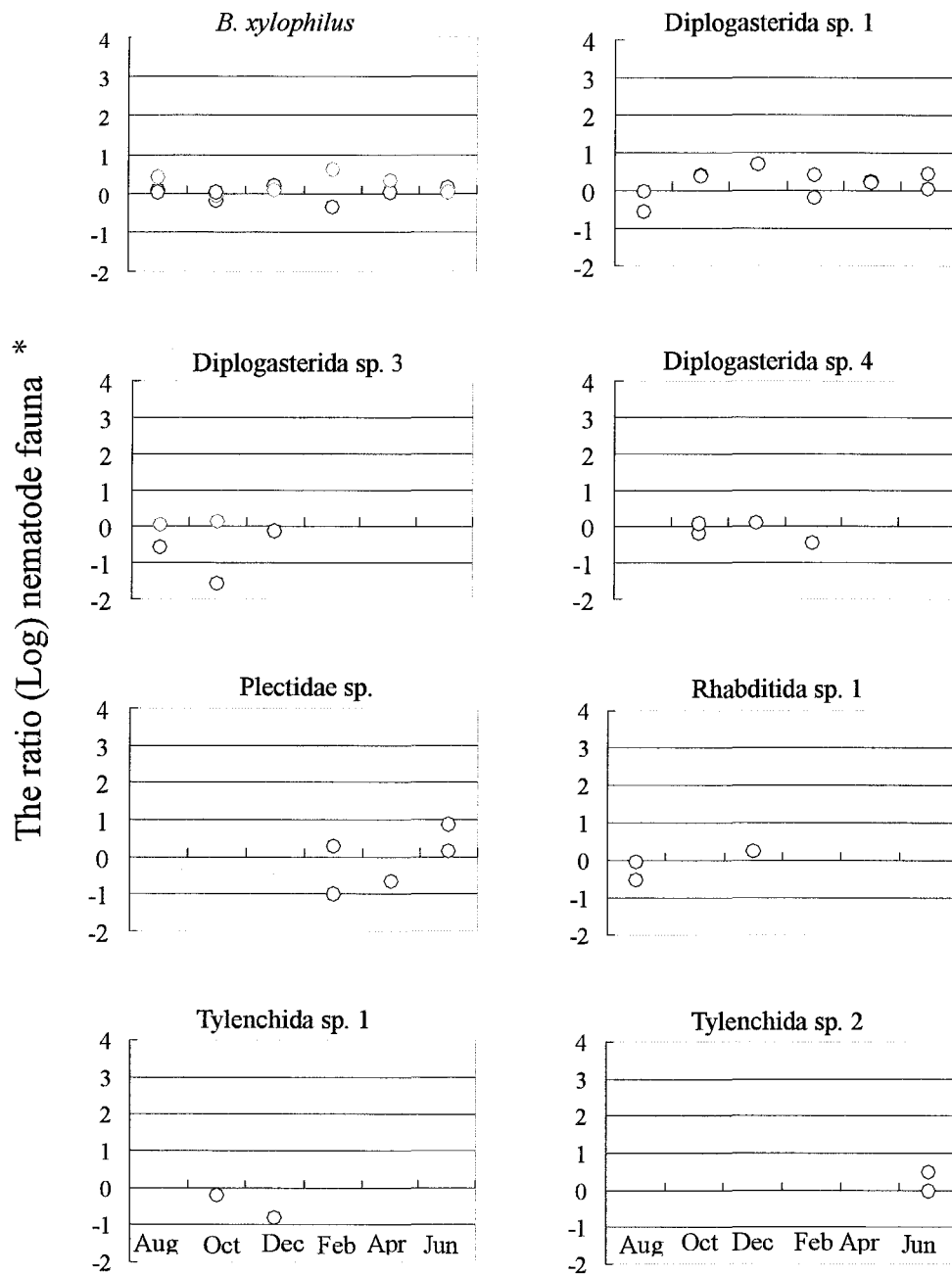
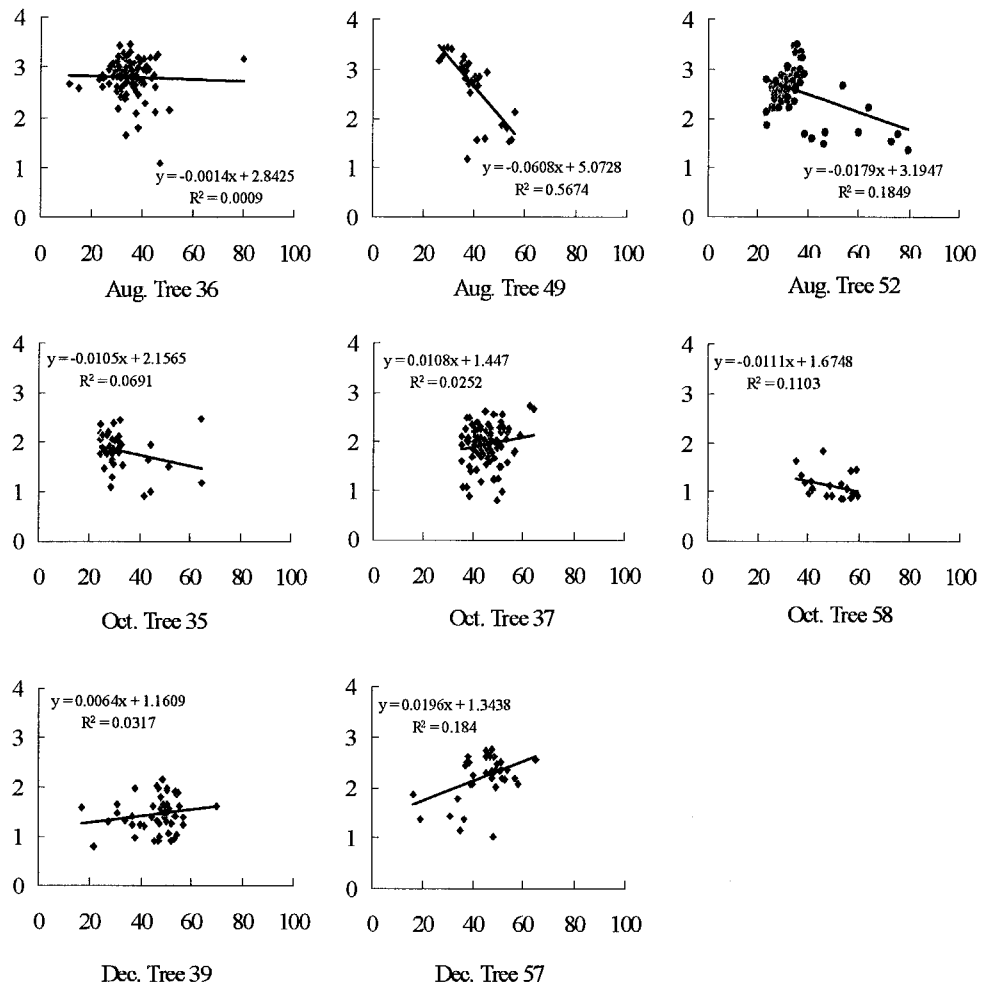


Fig. 3. Effect of pupal chambers of *M. alternatus* on the population density of dominant nematode species.*The ratio of nematode numbers in the wood samples including PC and/or other tunnel fractions to that in the wood samples without them.

Log (Number of *B. xylophilus* /gr dry wood +1)



Water content (%)

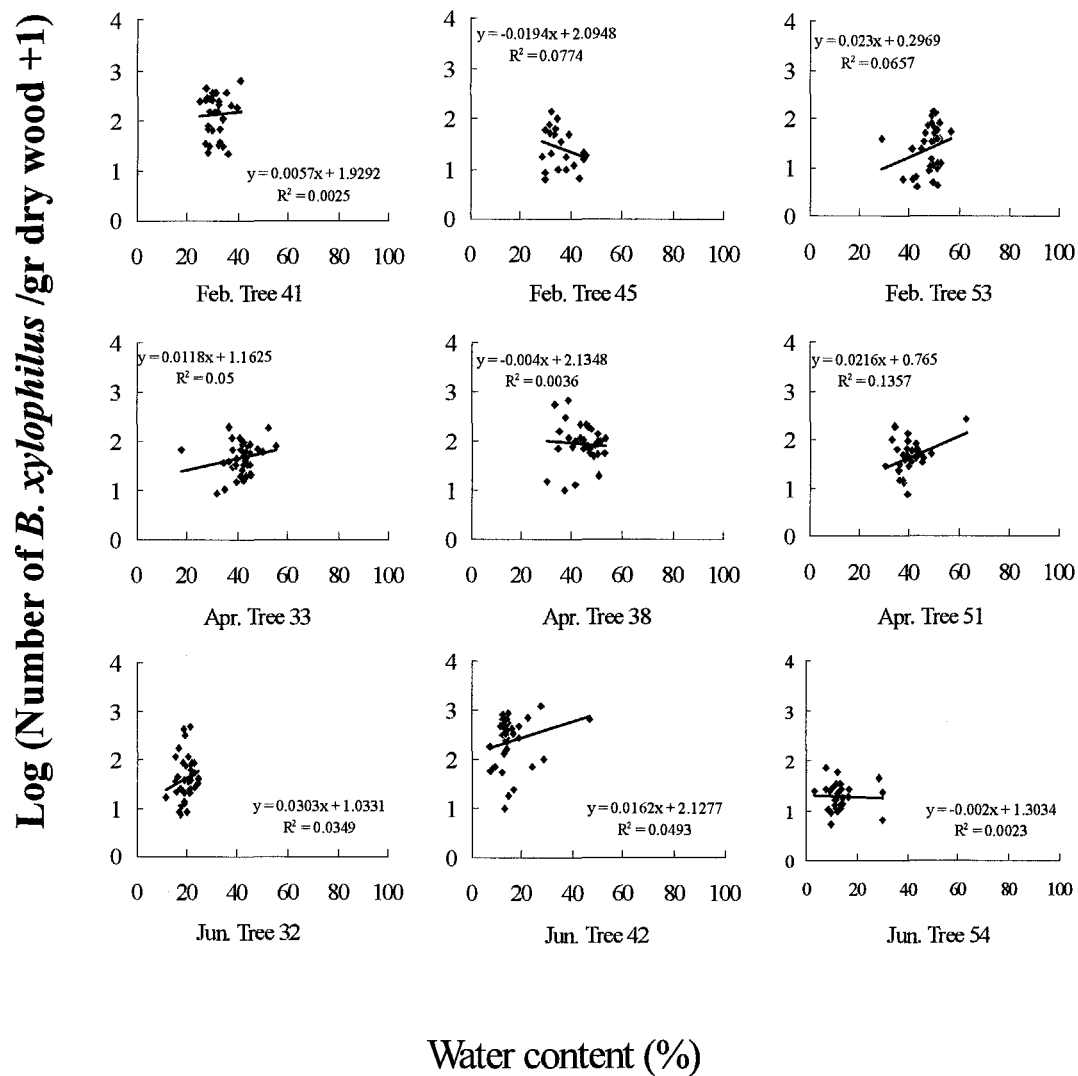


Fig. 4. Relationship between the number of *B. xylophilus* and that of water content on dead pine trees.

Braasch is very similar to ours in that it has an indistinct anus. Further studies are needed to clarify its phylogenetic relationship with other nematodes.

The number of the PWNs decreased in December than increased in February, and then decreased again until June when the experiment terminated. Some trees contained large PWN populations in June. Fukushige and Futai (1987) reported a similar fluctuation in the number of *B. xylophilus* which decreased from October to December and increased from December to February and there was no correlation between nematode numbers and host water content, as we found here (Fig. 4).

Futai *et al.* (1986) studied population changes of both *B. xylophilus* and other free-living nematodes in insecticide-treated and non-treated pine logs. Their studies showed that the population density of *B. xylophilus* was positively correlated with that of the free-living nematodes from November to the following January, while the number of the free-living nematodes decreased from October to November, then increased again in February. The present study examined the correlation between each of the free-living species and *B. xylophilus*. The population change of Diplogasterida sp. 1. on each tree was related to that of *B. xylophilus* over the experimental period (Fig. 1). At the microhabitat level, however, consistent correlation patterns were rare between *B. xylophilus* and any nematodes. These results suggest that there might be a positive relationship between *B. xylophilus* and Diplogasteridae sp. 1, but distribution of either nematodes among wood pieces of wood pieces (2 x 1 x 1cm) were more or less at random. Consequently, it was not possible to detect any relationship between them at the microhabitat level.

Kanzaki *et al.* (2002) isolated both *Rhabdontolaimus psacothaeae* Kanzaki and Futai (Diplogasterida: Diplogasteridae) and *B. conicaudatus* Khanzaki, Tsuda and

Futai from adults of the yellow-spotted longicorn beetle, *Psacothaea hilaris* Pascoe (Coleoptera: Cerambycidae) from fig trees, *Ficus carica* L. Kanzaki and Futai (2002) also found these two nematodes to be sympatric in their vector's body and in their host fig trees. Differences in their food preference in host trees and in the part of the vector body must enable *R. psacothaeae* and *B. conicaudatus* to be sympatric. The present study, also found that, a large population of both Diplogasterida sp. 1 and PWN congregated around both PC and tunnels of *M. alternatus*, perhaps difference in feeding habit of these two nematodes enabled them to be sympatric.

In natural stands, *B. xylophilus* is carried from wilt-killed to healthy pines by *M. alternatus* (Mamiya and Enda, 1972; Morimoto and Iwasaki, 1972). *M. alternatus* PCs are the most important places for PWN in infested trees due to blue stain fungi and some fungal flora. When the number of the PWNs that aggregate around the PCs of *M. alternatus* in wilt-killed pines are high, the number of PWNs carried by the beetles emerging from the PCs increase, Maehara *et al.*, 2005). Kobayashi *et al.* (1974, 1975), Fukushige (1991) and Maehara and Futai (2000) reported that the blue-stain fungi which prevailed around PCs would serve as food for *B. xylophilus*. Further, intense blue-stain on the walls of *M. alternatus* PCs increased the number of PWN aggregating around these PCs (Maehara *et al.*, 2005). In our study, several mycophagous nematodes such as species of Tylenchida, two species of *Bursaphelenchus* and PWN were detected, but only PWN preferred to aggregate around PC. In most cases, however, not only mycophagous PWN but also saprophagous Diplogasterida sp. 1 was more abundant around PC and tunnels than elsewhere in the wood. PC and tunnels might provide suitable humidity and sufficient nutrients for growth and reproduction of fungi and other microbes, including food for nematodes. These field studies suggested that many factors

that could affect the micro-climate of PCs and tunnels. Environmental conditions and other fungal species besides blue-stain fungi might play an important role in providing suitable conditions for propagation of the PWN. *Monochamus* beetles should introduce many organisms, including various fungi into pine trees. This study focused on the nematode fauna, but in Chapter III, I clarify the exact relationship between PWNs and the co-inhabiting fungi in dead pine tree.

CHAPTER II

Nematode species inhabiting pine trees killed by the pinewood nematode, *Bursaphelenchus xylophilus*.

Introduction

Nematode fauna inside pine trees should be closely related to the bark beetles. In general, bark beetles are considered to be the most destructive insects in forests (Berryman, 1974). Bark beetles attack trees roughly in two manners; firstly the beetles cause damages by making their galleries and thereby devalue the timbers, and secondly they may introduce microorganisms into the trees, including nematodes and blue stain fungi. Among the nematodes associated with bark beetles, relatively important species are belonging to the subfamily Neotylenchoidea, including the genera *Parasitylenchus*, *Contortylenchus*, *Sphaerularia* and *Allantonema* (Messey, 1974).

Some nematodes establish phoretic associations with insects, in which the nematodes use the insect only as a vector carrying them to a new environment without any trophic associations. Genus *Bursaphelenchus* is typical of this group of nematodes. Many *Bursaphelenchus* spp. inhabit and are carried by the insects such as scolytid and cerambycid beetles (Hunt, 1993).

Banage (1963) concluded that the feeding habits of nematodes could be categorized by the environmental conditions of their habitat. The most dominant group is plant feeders on vegetation or under the soil, and the next group is microbial feeders. The omnivorous feeders and the predatory Mononchida are much less in number.

In the previous chapter, I reported that 15 nematode species were inhabiting in pine trees killed by PWN. In this chapter, the bionomics and the general characters of these species will be described in detail.

Materials and Methods

NEMATODE EXTRACTION

Wood samples of dead pine trees were collected in August, October, December 2004, February, April and June 2005 as mentioned in Chapter I. Wood pieces were sliced into small chips with pruning shears and placed in a Baermann funnel for 40 hours to extract nematodes. After extraction, the chips were dried overnight at 60°C to determine their dry weight.

FIXING AND MOUNTING

The nematodes extracted were killed in water bath at 70 – 80°C for 2 min and fixed in TAF (triethanolamine 2 ml; formalin 7 ml; distilled water 91 ml). Fixed nematodes served for light microscopic (LM) observations. For observations by LM, the nematodes were processed in quick method of transferring to glycerine by processing through warm lactophenol (Siddiqi, 1964), rapid method as modified by De Grisse (1969), mounted on permanent slides and observed under LM. All measurements were made using a drawing tube attached to a Nikon Eclipse E600 light microscope, Nikon, Japan.

SCANNING ELECTRON MICROSCOPY (SEM)

SEM was conducted to observe only the new species of *Bursaphelenchus*. For SEM, fixed nematodes were transferred into vial ($\Phi = 10\text{mm}$) that contained a drop of glycerin. Distilled water was added drop by drop until whole nematodes were immersed in. Nematodes were transferred to a drop of formalin 4%. The nematodes then were post-fixed in 2% osmium tetroxide for 12 hours at 25°C following the methods described by Nguyen and Smart (1995). Dehydrated through a gradual ethanol concentration gradient 20% (over night), 30%, 50%, 75%, 95% (3 hours) and 99.5% (overnight) at 25°C, and then were critical point dried with liquid CO₂, mounted on aluminum stubs, coated with gold (200 nm thickness), and observed using a KEYENCE VE 8800 scanning electron microscope, Keyence, Japan.

NEMATODE CLASSIFICATION

Nematodes were classified based on their morphological characters at order, family and genus level. Nematodes with stylet and large median bulb were classified as aphelenchids (plant, fungal and lichen feeders); with a relatively strong stylet and less obvious median bulb as tylenchids (plant parasites, lichen feeders and a few fungal feeders); with narrow stoma and three-part oesophagus as rhabditids and areolaimids (bacterial feeders); with median bulb but lacking spear and stylet, some showing mouth cavity with dorsal teeth as Diplogasterids (bacterial feeder and saprophagous); and with mouth cavity in barrel shape and strongly sclerotized, with one large dorsal tooth and at opposite of it usually an ridge, oesophagus base without vesicles were as Mononchids (predator); terminal bulb absent, mouth cavity tubular, absent aphid spiral shape or circular as Monhisterids (saprophagous).

Remarks

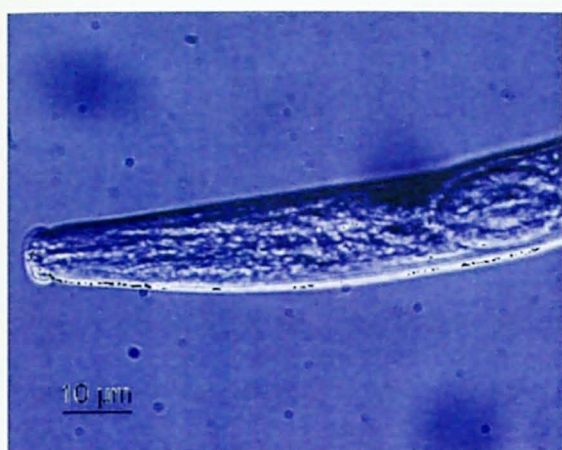
1. *Bursaphelenchus xylophilus*

BIONOMICS:

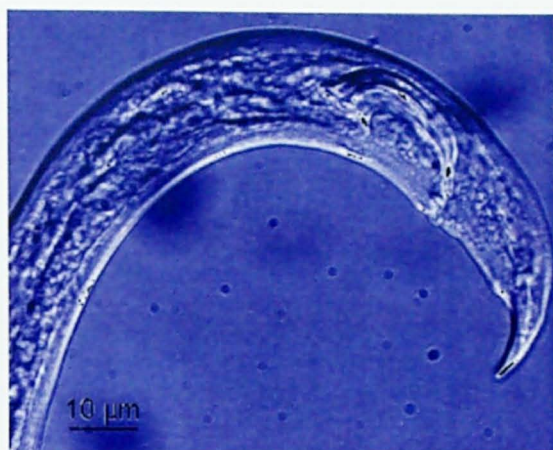
B. xylophilus is known as the 'pine wilt', or 'pinewood' nematode because of the symptom induced in susceptible species of the genus *Pinus*. This nematode species is often found in the bark and wood of dead pine trees, as dispersal third stage or dauer larvae. *B. xylophilus* is commonly vectored by the longhorn beetles, *M. alternatus* and causes a serious wilt disease of pine trees, but also feeds on fungi in dead trees (Hunt, 1993). *M. alternatus* is thought to be a major vector in Japan because it is most frequently associated with dying pine trees and is always heavily infested with these nematodes (Kobayashi *et al.*, 1984). Numerous beetles of various genera in the family Cerambycidae, Curculionidae also colonise recently-killed pines (Mamiya and Enda, 1972; Morimoto and Iwasaki, 1972; Linit *et al.*, 1983; Linit, 1988).

GENERAL CHARACTERS

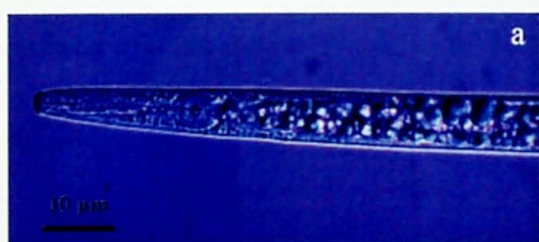
Cephalic region high, offset, separated into six lips. Stylet small and basal swelling. Oesophageal gland lobe slender and about three to four body widths long. Vulva posterior, the lip anterior over hanging to form a flap. Genital tract monoprodelfic. Tail sub-cylindroid with a broadly rounded terminus. Mucro usually absent, except for some population. Spicule large and strongly arcuate, the apex bluntly rounded, and the rostrum prominent and pointed. Tail arcuate with pointed, talon-like terminus bearing a small bursa, tail spike and just anterior to the start of the bursa (Fig. 5-A; Hunt, 1993). All these observations were consistent with the previous studies.



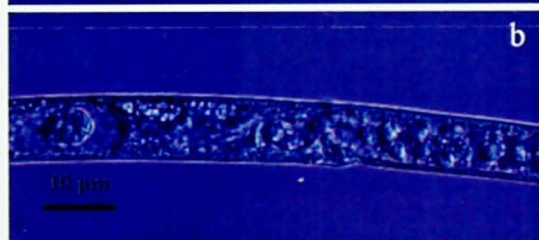
1



2



a



b

3



4

Fig. 5-A. *Bursaphelenchus xylophilus*.

1. Male head; 2. Male tail; 3a. Female head; 3b. Vulva; 4. Female tail.

2. *Bursaphelenchus* n. sp.

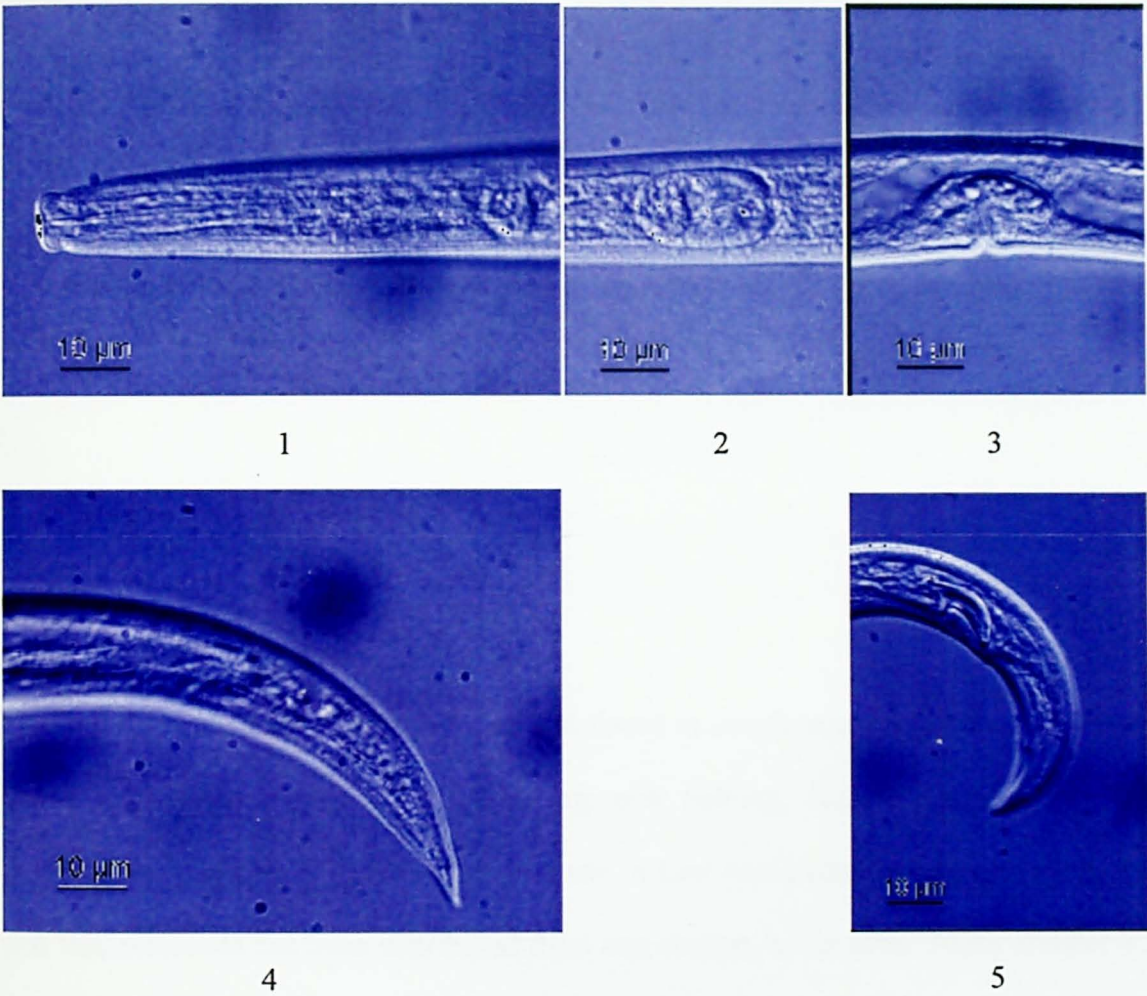
BIONOMICS

A new species of the genus *Bursaphelenchus* was found and described here as *Bursaphelenchus* n. sp. The nematodes belonging to the genus *Bursaphelenchus* have been known as phoretic nematodes associated with bark beetles. Therefore, the vector beetle plays an important role for the nematode distribution; the specific beetle should bring the specific nematode in the specific season. Under this study, this new *Bursaphelenchus* species had been found just in December and June. To determine its vector beetle further studies are needed.

GENERAL CHARACTERS

Body slender, cylindrical, annulated, ventrally curved when killed by heat. Cuticle marked by fine transverse width striae. Lateral fields with three incisures. Lip region offset, separated into six lips, two lateral lip sectors narrower than the other four. Stylet slender, lacking basal knob. Median bulb elongate-oval, longer than width, one body diameter length. Valves well developed, plate not central but posterior to the middle of median bulb. Ovary single, outstretched, oocytes arranged in double rows, sperm visible in oviduct. Vulva not protuberant, vulval flap absent. Post uterine sac short, less than one body-diam. long. Rectum and anus, probably non-functional, difficult to distinct. Intestine terminating as blind sac. Tail tapering to conoid with pointed terminus. Male spicule arcuate, condylus rounded, slightly elongated, sometime recurved dorsally, lamina smoothly and symmetrically curved, cucullus small, flattend. Tail strongly ventrally curve, pointed tip, spade-shaped terminal bursa is difficult to see

under LM but clearly distinguished under SEM. Two pairs of caudal papillae present: one just pre-anal pair and one ventral post-anal pair in front of the terminal bursa (Fig. 5-B).



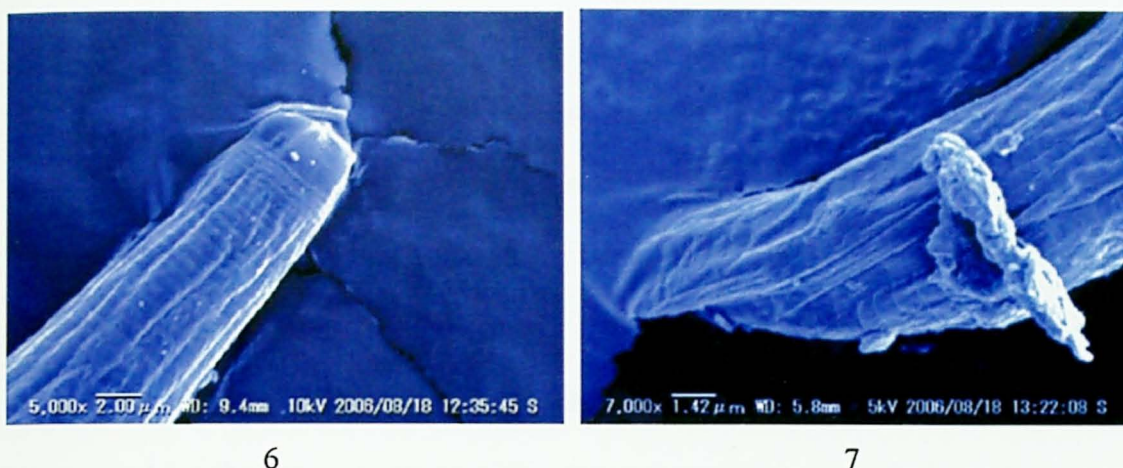


Fig. 5-B. *Bursaphelenchus* n. sp.

1. Female head; 2. Median bulb; 3. Vulva; 4; Female tail; 5. Male tail; 6. SEM of male head; 7. SEM of bursa.

3. *Bursaphelenchus sinensis*

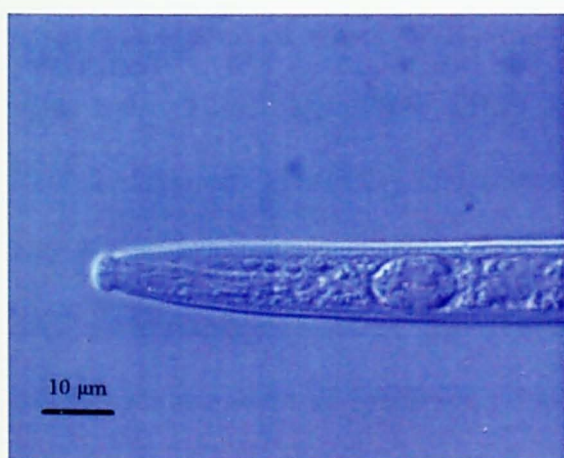
BIONOMICS

Bursaphelenchus sinensis was first found in coniferous wood introduced from China as packaging material into Liefering, near Salzburg, Austria (Palmisano *et al.*, 2004). This nematode is supposed to originate in East Asian countries and it is possible that this nematode has been distributed from one country to the other Asian country by their vector beetles.

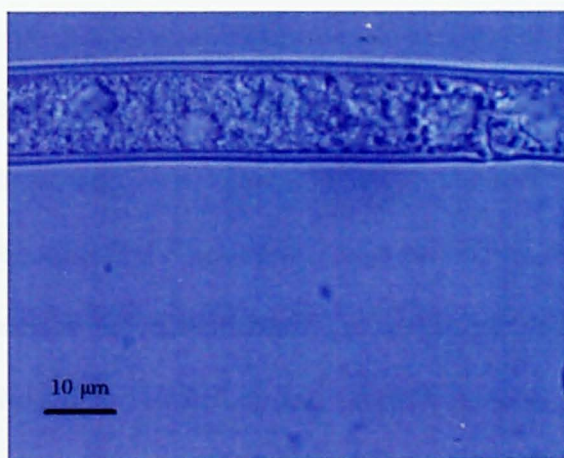
This nematode species was obtained from wood pieces of dead pine trees killed by PWN, as is consistent with the bionomics of *B. xylophilus* and *Bursaphelenchus* n. sp in general. Their populations were very few, which made it difficult to obtain more samples and to cultivate its culture.

GENERAL CHARACTERS

General characteristics of the genus *Bursaphelenchus* were found in this species. Cephalic region high, offset. Stylet small, basal swelling. Oesophageal gland lobe slender. Vulva posterior. Genital tract monoprodelfic. Female tail slightly sharp. Spicule capitulum compact, rostrum and condilus fused. A specific character of *B. sinensis* was also found in this species. Relatively small body length. The male spicules are unusual for the genus, separate with a squared condylus, ventrally bent, rostrum not clearly differentiated. Female with small vulva flap, tail conical, almost straight, terminus almost pointed sometimes with a small mucro (Fig. 5-C). Based on the spicule structure, this species belongs to aberans group in genus *Bursaphelenchus* (Ryss, 2005). After carefully checked by a Japanese nematologist, this species proved to be *Bursaphelenchus sinensis*, which has been isolated from Kyoto and Ibaraki prefecture, Japan (Kanzaki, pers. comm.)



1



2

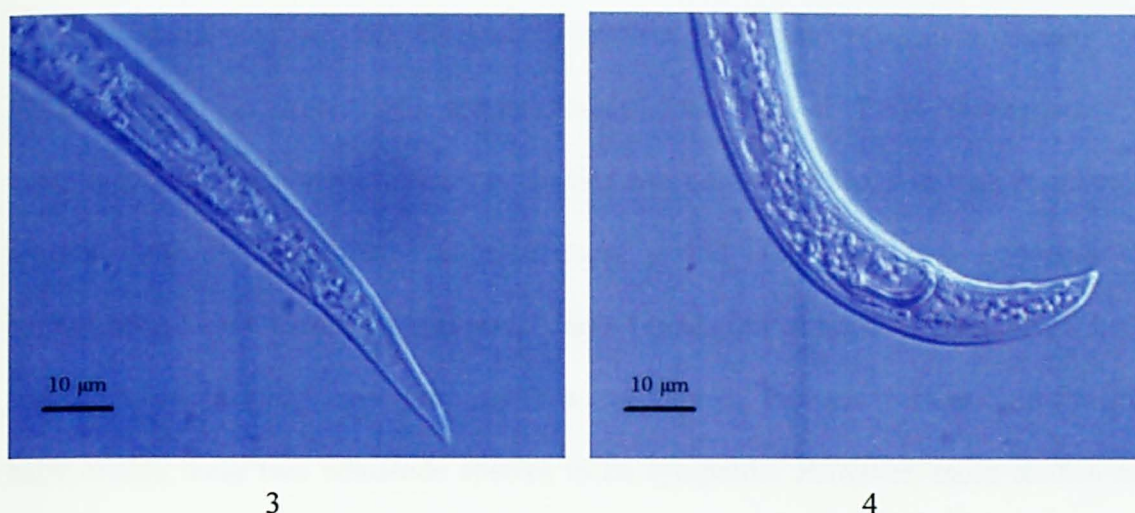


Fig. 5-C. *B. sinensis*.

1. Female head; 2. Vulva; 3. Female tail; 4. Male spicule.

4. Diplogasterida sp. 1

BIONOMICS

A species of the order Diplogasterida was extracted from bark and wood of pine trees killed by PWN. This species supposedly belong to the genus *Rhabdontolaimus*. Generally, *Rhabdontolaimus* spp. are known to have relationship with coleopteran insects. Previous studies reported that *Psacothaea hilaris* carried both *Rhabdontolaimus psacothaeae* and *Bursaphelenchus conicaudatus*, a mycetophagous commensal nematode. *R. psacothaeae*, therefore, shares host tree and vector beetles with *B. conicaudatus* (Kanzaki and Futai, 2002; Kanzaki *et al.*, 2002).

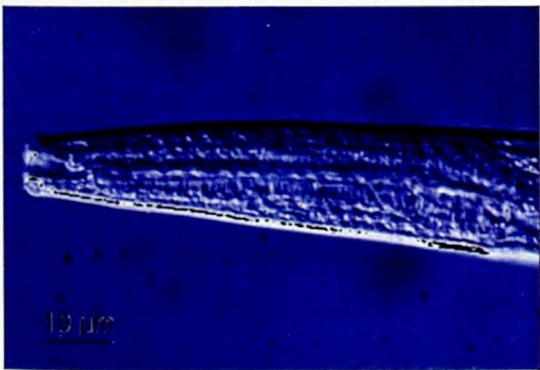
The results in the Chapter 1 showed that the population density of *Diplogasterida* sp. 1 changed synchronously with that of PWN throughout the experimental period. Interestingly, this species was constantly found in high population together with *B. xylophilus* over experiment period. Table 1 shows frequency of appearance of each nematode species. A large population of both *Diplogasterida* sp. 1 and *B. xylophilus* congregated around PCs and tunnels. Perhaps difference in feeding habit enable these two nematode species to be sympatric. However, more studies are needed to examine alternative possibility as for feeding habit of *Diplogasterida* species, because some studies clarified *Diplogasterida* to be predator species e. q., Bigrami, (1989).

GENERAL CHARACTERS:

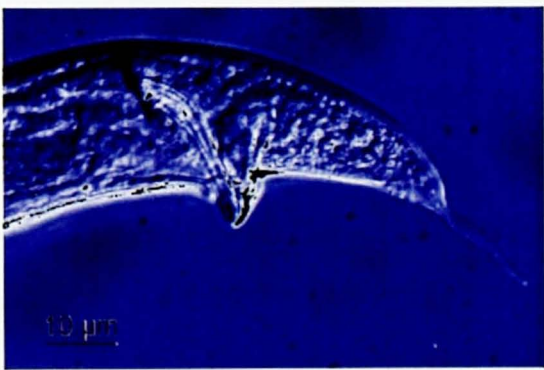
Diplogasterida sp. 1 (*Rabdontolaimus* sp.) is characterized by stoma about three times longer than width. Rhabdion heavily sclerotized. Dorsal meta rhabdion with denticular ridges bearing varying number of teeth. Vulva median, ovaries paired and opposed, female tail conoid to sharply rounded or filiform terminus. Spicule paired, ventrally arcuate (Fig. 5-D; Messey, 1974).

Table 1. Frequency of appearance of each nematode species over experimental period.

	Aug	Oct	Dec	Feb	Apr	Jun
<i>B. xylophilus</i>	65.9	61.5	37.6	41.1	64.4	38.0
<i>Bursaphelenchus</i> n. sp.	0.0	0.0	2.1	0.0	0.0	1.8
<i>B. sinensis</i>	0.0	0.0	0.0	0.9	1.2	0.7
Diplogasterida sp. 1	30.6	40.1	21.5	32.7	54.0	9.1
Diplogasterida sp. 2	11.0	0.0	0.4	0.0	0.0	0.0
Diplogasterida sp. 3	34.1	33.2	12.2	0.0	0.0	0.0
Diplogasterida sp. 4	0.0	8.4	7.2	4.2	0.0	0.0
Monhysterida sp. 1	0.0	1.5	0.0	0.0	0.0	1.1
Monhysterida sp. 2	0.0	0.4	0.0	0.0	0.0	0.0
Mononchida sp.	0.0	0.0	1.7	0.0	3.1	0.0
Rhabditida sp. 1	21.5	0.0	4.6	0.0	0.0	0.4
Rhabditida sp. 2	0.0	1.1	0.0	0.0	0.0	0.0
Plectidae sp.	0.0	0.0	10.5	7.0	8.0	3.3
Tylenchida sp. 1	0.0	26.0	13.9	6.5	0.0	0.0
Tylenchida sp. 2	0.0	0.0	0.0	0.0	0.0	15.7



1



2

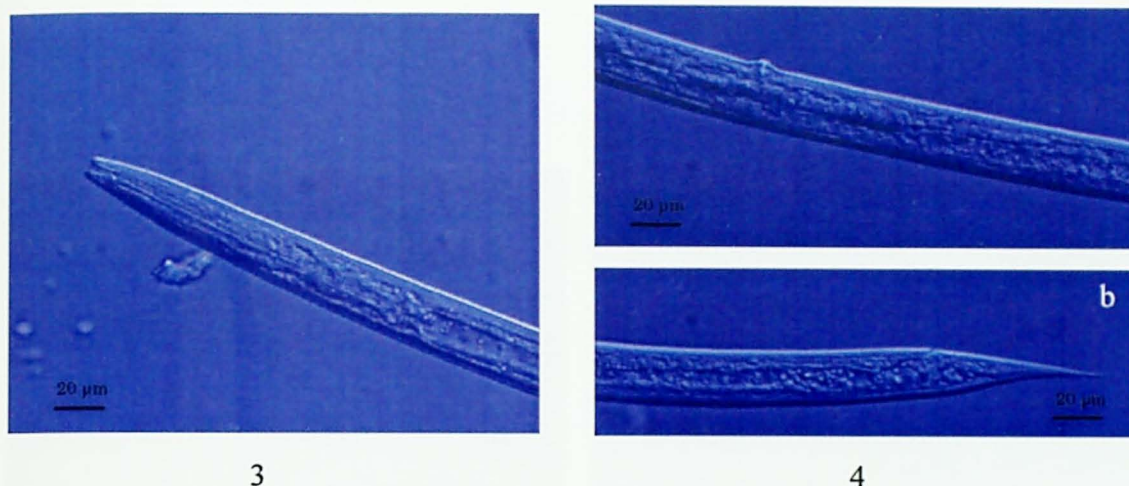


Fig. 5-D. *Diplogasterida* sp. 1

1. Male head; 2. Male spicule and tail; 3. Female head; 4a. Female vulva; 4b. Female tail.

5. *Diplogasterida* sp. 2

BIONOMICS:

The bionomics of this species was almost similar to that of *Diplogasterida* sp 1 (*Diplogasterida*), though the number was very few and the distribution was very sparse compared with *Diplogasterida* sp. 1.

GENERAL CHARACTERS:

A little information is available for the description of this species due to limited number of the specimen collected. Based on the characters, however, it was not difficult to identify it as *Diplogaseterida* species. That is, spear and stylet absent, median bulb present, and mouth cavity with dorsal teeth found in some individuals. In addition, the distinctive characters of this species are short stoma and short male tail with variously formed terminus, i.e. sharp to round or filiform (Fig. 5-E). Spicule paired, relatively

arcuate.

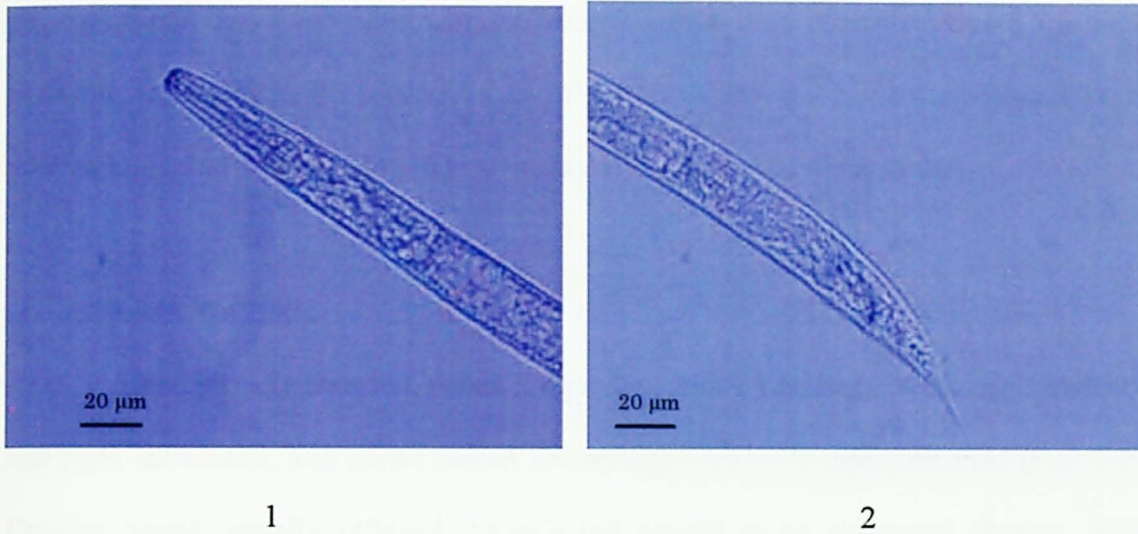


Fig. 5-E. *Diplogasterida* sp. 2

1. Male head; 2. Male tail.

6. *Diplogasterida* sp. 3

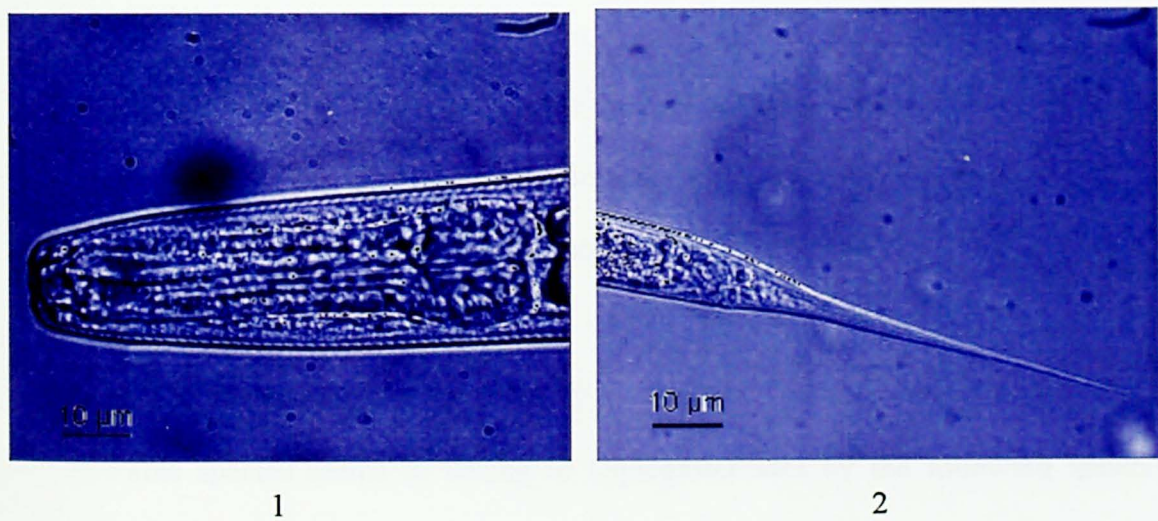
BIONOMICS:

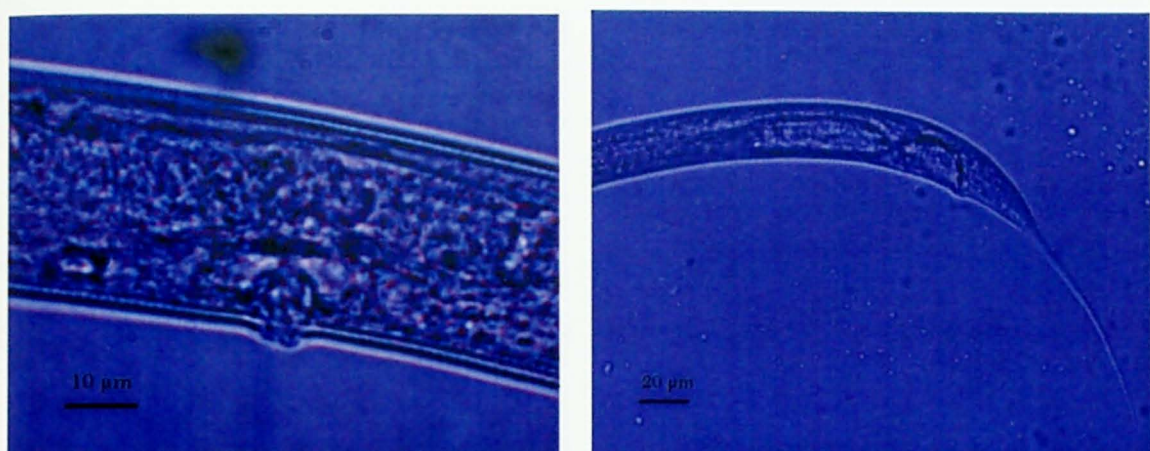
Based on their characteristics, this species might be a species of the genus *Micoletzkyia*. Almost all of *Mikoletzkyia* species are known to be associated with bark beetles of pine trees (Messey, 1974). Under natural conditions, various beetles carry many species of nematodes, though phoretic relationship must be specific between beetles and nematodes. Because this experiment was conducted in the natural field, various beetles should have flied to the PWN-killed pine trees, and some species of the beetles might have introduced this nematode into the trees then Massey (1974) reported that some members of genera *Mikoletzkyia* were commonly carried by beetles. They

were recovered from bark infested with beetles, but they were also inhabitants of the outer bark feeding on lichens. It does not seem to be a true inhabitant of bark beetle galleries since they have often been collected from the bark of control trees. It is most probable for them to be carried by other group of insects. In this study, nematode species extracted from healthy trees were not counted in analytical studies.

GENERAL CHARACTERS:

Head broadly rounded, stoma longer than width, two large teeth, one dorsal and one right subventral, and gonad paired. Oesophagus typically diplogasteroid (Fig. 5-F). Ovaries paired, usually reflexed. Female tail conoid to an elongated (Ruhm, 1959; Messey, 1974)





3

4

Fig. 5-F. *Diplogasterida* sp. 3.

1. Female head; 2. Female tail; 3. Vulva; 4. Male tail

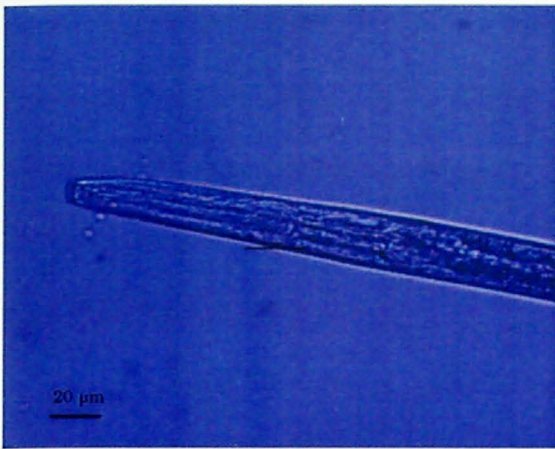
7. *Diplogasterida* sp. 4.

BIONOMICS:

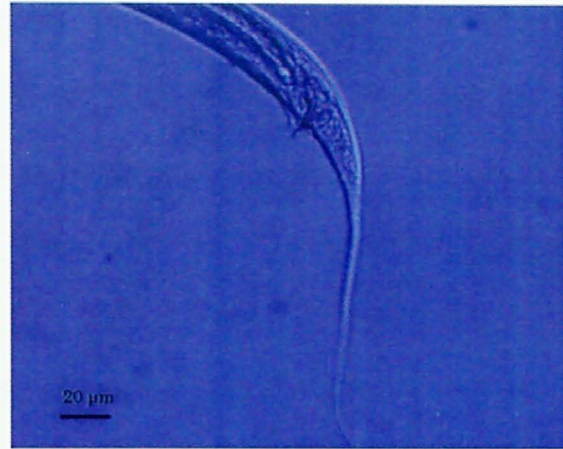
A few numbers of this species of nematodes were collected from bark and wood pieces of samples. Therefore, little information is available for discussion. This nematode species were collected from October to February (Table 1).

GENERAL CHARACTERS:

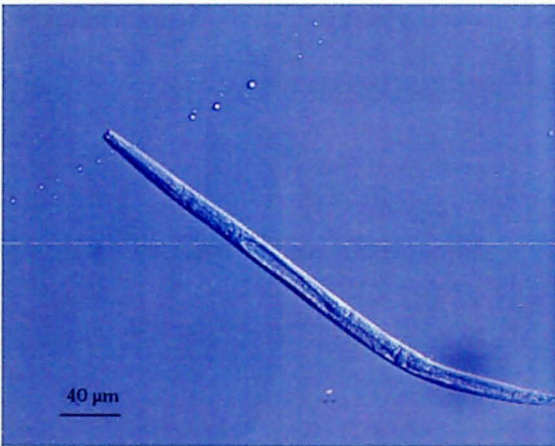
This species seems to belong to *Diplogasteroides* by the following general characteristics of *Diplogasteroides* species. Tail was usually long and filiform. Stoma about three times longer than the width, rhabdion heavily sclerotized, spicule paired, ventrally arcuate, several pairs of caudal papillae (Fig. 5-G). Bursa rudimentary or absent (Messey, 1974). Detailed information is not possible due to the shortage in numbers of specimen available.



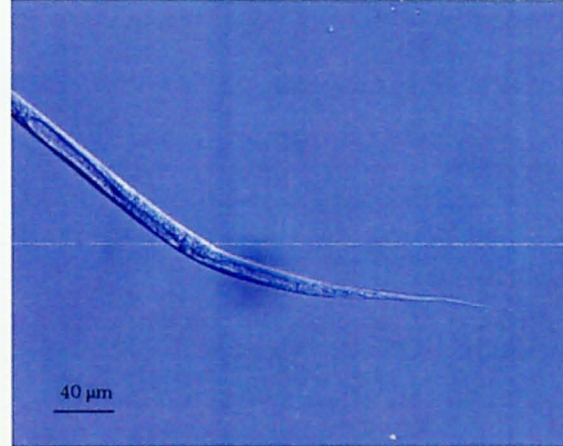
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4

Fig. 5-G Diplogasterida sp. 4.

1. Male head; 2. Male tail; 3. Anterior part of female; 4. Posterior part of female

8. Monhysterida sp. 1

BIONOMICS

Nematodes belonging to order Monhysterida are saprophagous and usually inhabit marine or fresh water (Coomans *et al.*, 1996). Interestingly, we found these

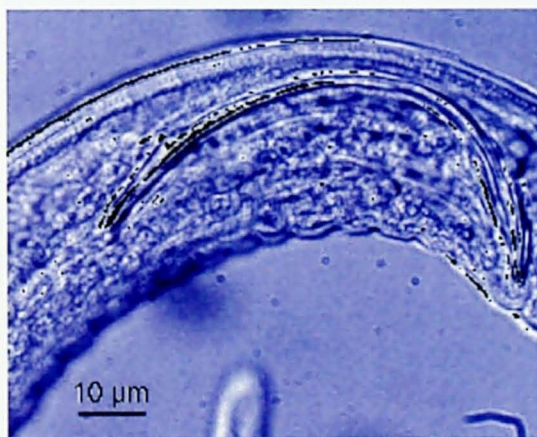
free-living nematodes in dead pine trees, though the factors of their distribution could not be clarified yet. They might be introduced into pine trees after the trees died, where many organisms harbored including bacteria and fungi.

GENERAL CHARACTERS

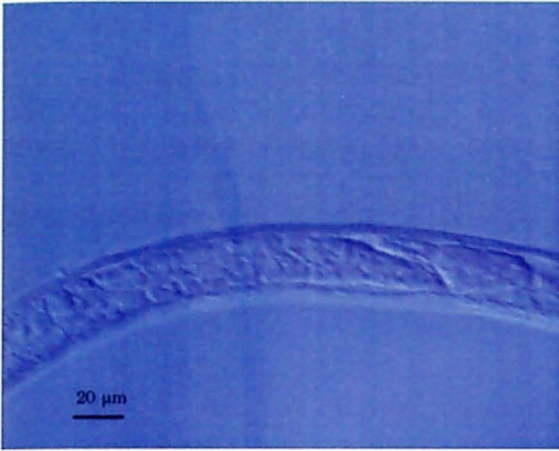
Terminal bulb absent, mouth cavity tabular, aphid spiral shaped or circular, as is consistent with the characters of Monhysteridae. The Monhysterida sp. 1 has some of the characteristics such as body elongated with rounded head and cylindrically tail with rounded tip. Spicule strongly curve and heavily sclerotized, male tail cylindrical. Female vagina heavily sclerotized (Fig. 5-H).



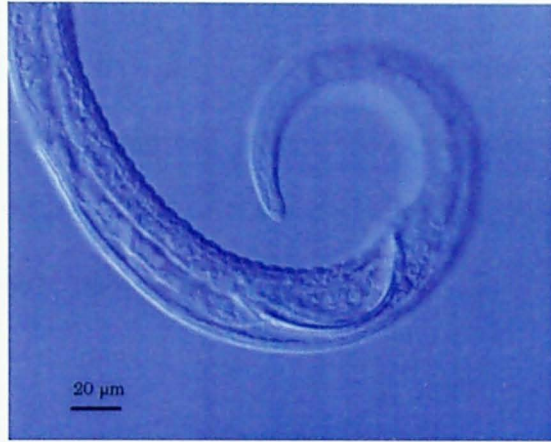
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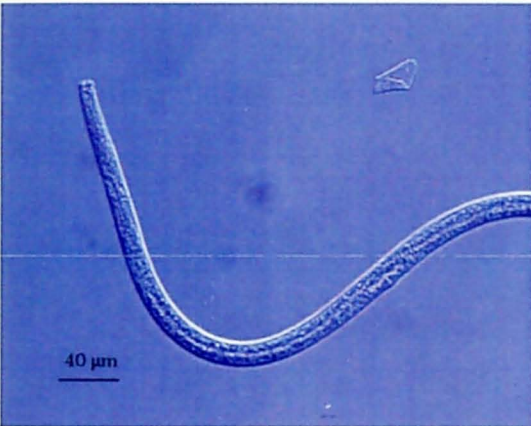
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Fig. 5-H. *Monhysterida* sp. 1.

1. Male head; 2. Male spicule; 3. Male testis; 4. Male tail; 5. Anterior part of female; 6. Posterior part of female.

9. *Monhysterida* sp. 2

BIONOMICS

Another species of *Monhysterida* was found in the present study. The distribution of this species were similar to *Monhysterida* sp. 1.

GENERAL CHARACTERS

Judging from the characters, i.e., terminal bulb absent, mouth cavity tabular or absent, not barrel shaped, aphid spiral shaped or circular; this species belongs to Monhysterids group. Monhysterida sp. 2 is distinguished from Monhysteridae sp 1 by vulva position, that is, about 60% Vs 76% from total body length (Fig. 5-I).

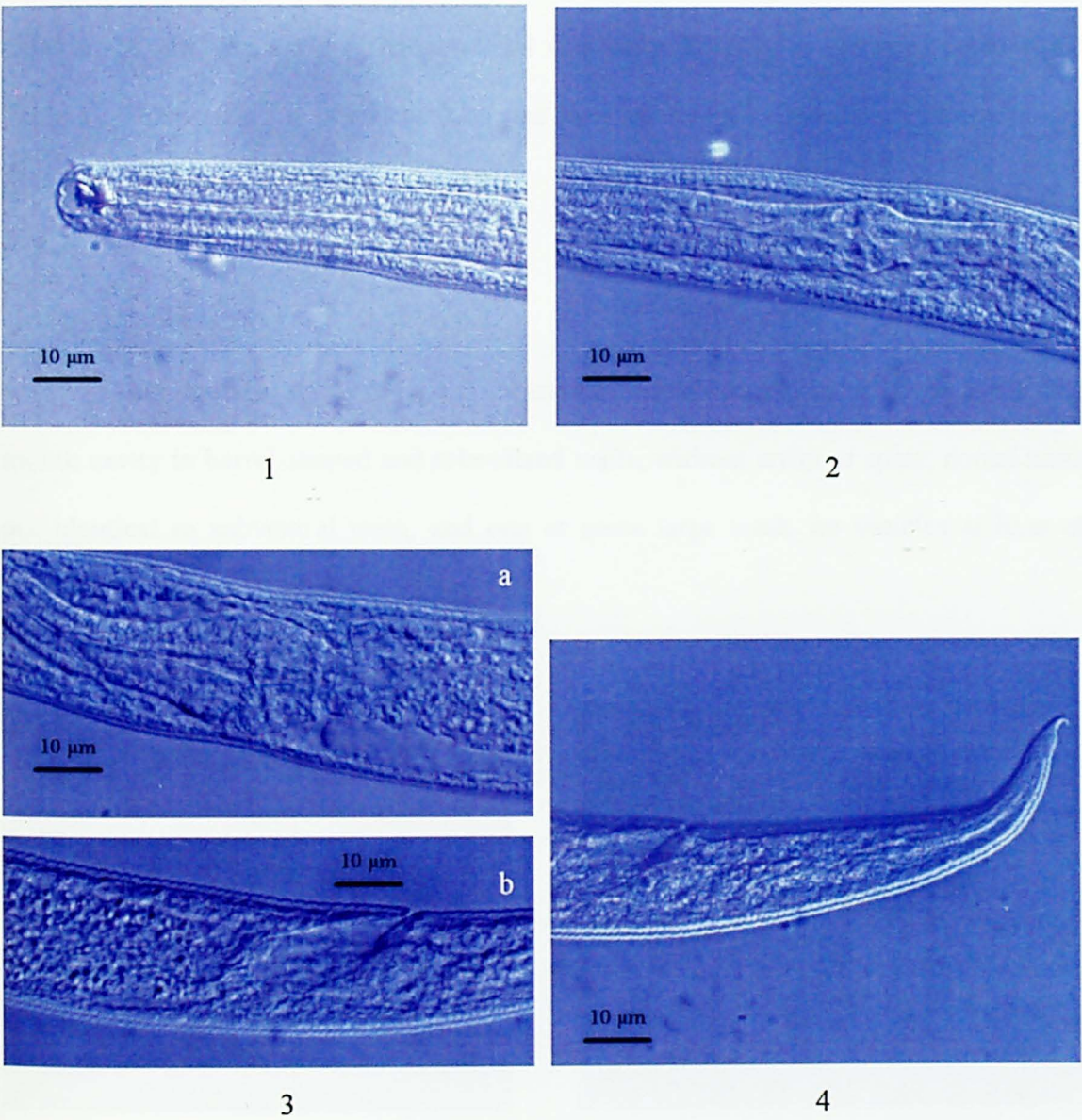


Fig. 5-I. Monhysterida sp. 2.

1. Female head; 2. Meta corpus; 3a. Basal bulb; 3b. Vulva; 4. Female tail.

10. Mononchida sp.

BIONOMICS

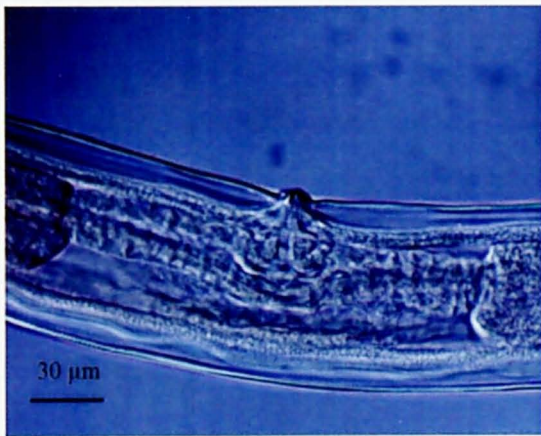
The Mononchida species is morphologically distinct from others because of its large body. Nematodes of Mononchida play the roles at three levels in forest ecosystem, i.e., predation, bacterial regulation, clay transport and decomposition, so they can be good indicators of changes of forest humus (Saur and Arpin, 1989). During the experiment, the nematode of Mononchida was obtained only in December and April (Table 1). The population of this species was more abundant in bark than in wood pieces of dead pine trees examined.

GENERAL CHARACTERS

This species showed typical characters of Mononchida, such as large and mouth cavity in barrel-shaped and sclerotized walls, without stylet or spear, dorsal tooth not identical to subventral teeth, and one or some large teeth, no vesicles at base of oesophagus. (Fig. 5-J; Bongers, 1988).



1



2

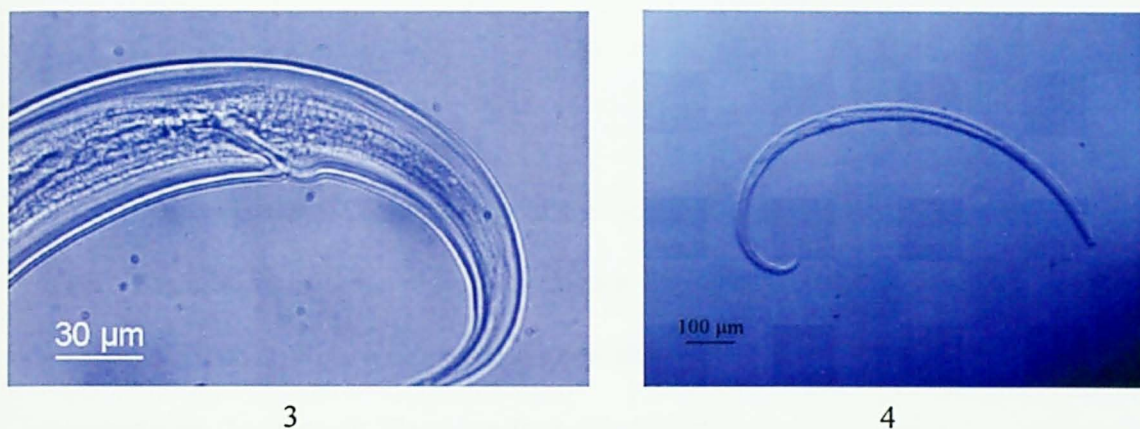


Fig. 5-J. Mononchida sp.

1. Female head; 2. Vulva; 3. Female tail; 4. Adult female.

11. Plectidae sp.

BIONOMICS

The genus *Plectus* of family Plectidae in order Aerolaimida is one of the most widely distributed and common nematodes in fresh water and terrestrial habitats, occurring all over the world. Species of Aerolaimida have been found mainly in fresh water, but also occur in wet soil. However, sometimes they occur in non aquatic, drier habitats (Coomans and Waele, 1983; Coomans *et al.*, 1996).

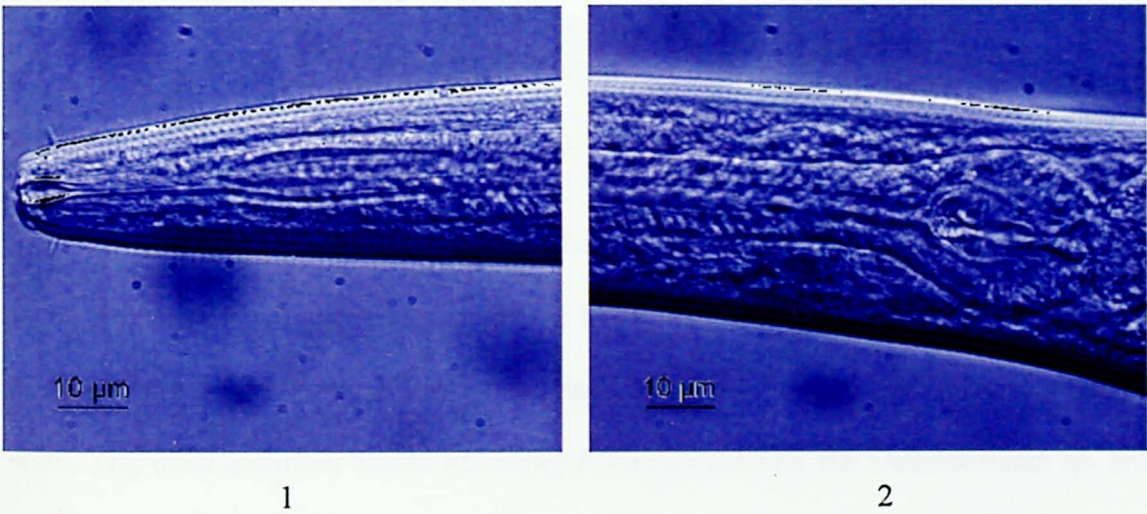
In this study, a large number of Plectidae species was first found in December 10.5% in frequency; see Table 1). Progress in wood decay must be a prerequisite factor in the distribution of terrestrial nematodes such as Plectidae species. After pine trees were killed by PWN, the bark and wood of the trees became softer and wetter. Under these conditions, the terrestrial nematode might migrate from soil to bark and stem of

trees.

GENERAL CHARACTERS

Plectidae and Rhabditidae share some morphological characters possibly because Rhabditidis originated from primitive Rhabditids under saprobiotic conditions (Lahl *et al.*, 2003). Observing the nematode obtained in the present study, some of the characteristics that discern Plectidae from Rhabditidae can be found in the head region shape and cuticularization of the mouth cavity, presence of bristles (setae) and chemoreceptors in the neck region (amphids; Fig. 5-K; Lahl *et al.*, 2003).

All of Plectidae that were isolated from dead pine trees inoculated with PWN were female, and no male has been detected so far.



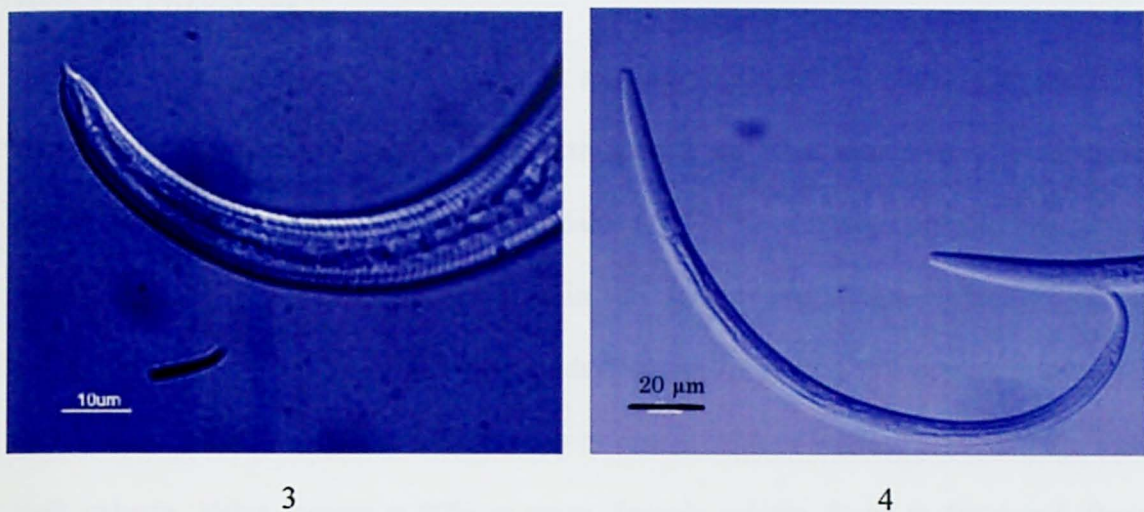


Fig. 5-K. Plectidae sp.

1. Female head; 2. Basal bulb; 3. Female tail; 4. Adult female.

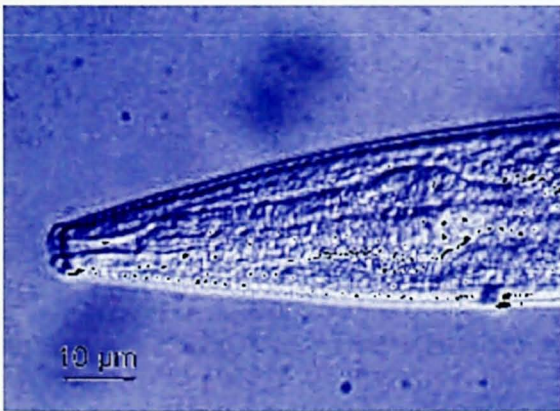
12. Rhabditida sp. 1

BIONOMICS:

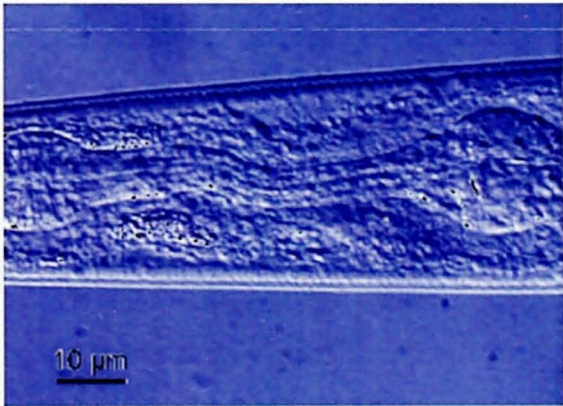
Nematodes belonging to order Rhabditida are common terrestrial nematodes. Some species inhabit soil usually as free living ones. Messey (1974) reported some species of the genus *Parasitorhabditis*, in Rhabditida associated of bark beetles in United State. The most common beetle vectoring *Parasitorhabditis* are *Dendroctonus* sp. and *Scolytus* sp. Rhabditida nematodes in the present study were collected from bark and wood pieces of pine samples and were recorded based on a few specimen. Therefore, available information to be discussed was very limited.

GENERAL CHARACTERS:

This species was identified as a species of Rhabditida due to the following morphological characters; stylet and spear absent, mouth cavity clearly wide, aphid hardly visible, and caudal gland absent. Males usually have well-developed caudal alae (Maggenti, 1991). The characters found in this species are similar to those of genus *Parasitorhabditis*; the lips angular to rounded, stoma consisting of elongate prorhadions, meso, meta and telorhabdions rudimentary or absent with or without median bulb, basal bulb valvate. Vulva locates at 90% or more. Spicule spicate, fused at distal end. Bursa peloderan with 8-12 pairs of bursa rays (Fig. 5-L). Based on these characters, this species might be classified as one species in the genus *Pararasitorhabditis*.



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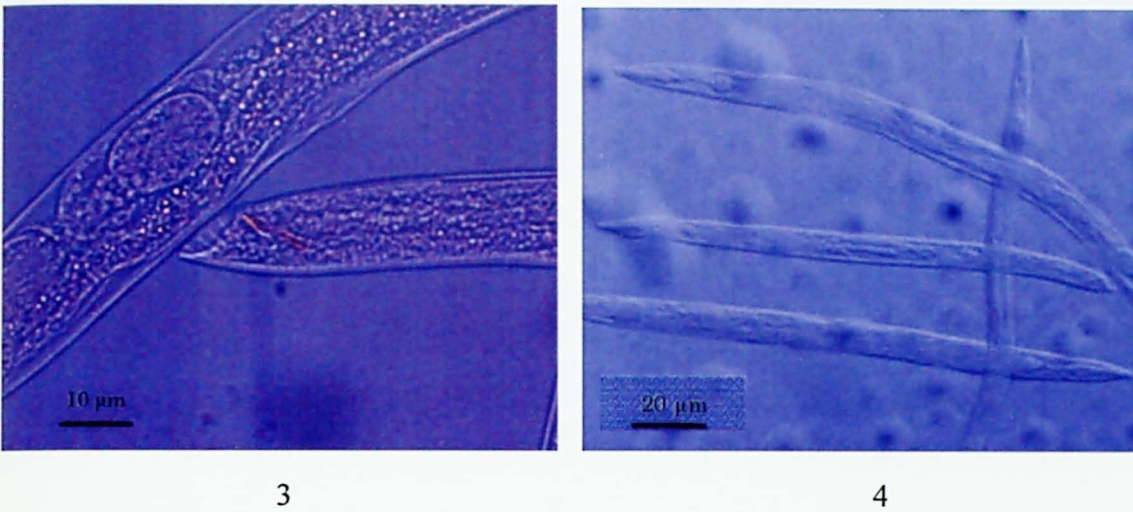


Fig. 5-L. *Rhabditida* sp. 1.

1. Female head; 2. Meta corpus and busal bulb; 3. Vulva and spicule; 4. Adult female and male.

13. *Rhabditida* sp. 2,

BIONOMICS

Generally, they showed similar bionomics to that of the *Rhabditida* sp.1, though specimen obtained were also limited.

GENERAL CHARACTERS

The morphological characters found in this species were almost same as *Rhabditida* sp.1, excepting that its stoma and corpus were longer than *Rhabditidae* sp. 1 (Fig. 5-M). Unfortunately, no male of this species was detected.

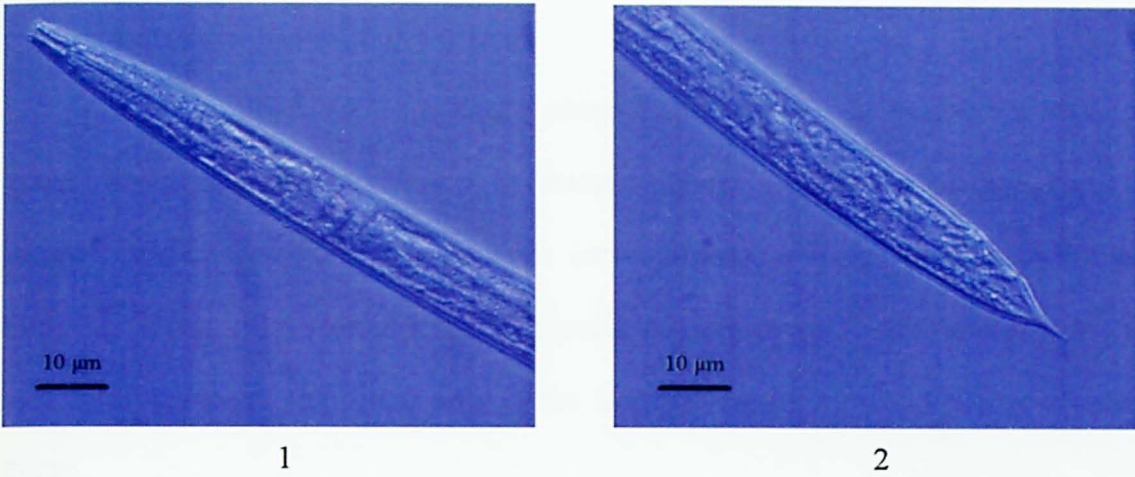


Fig. 5-M. *Rhabditida* sp. 2

1. Anterior part of male; 2. Posterior part of male.

14. *Tylenchida* sp. 1

BIONOMICS

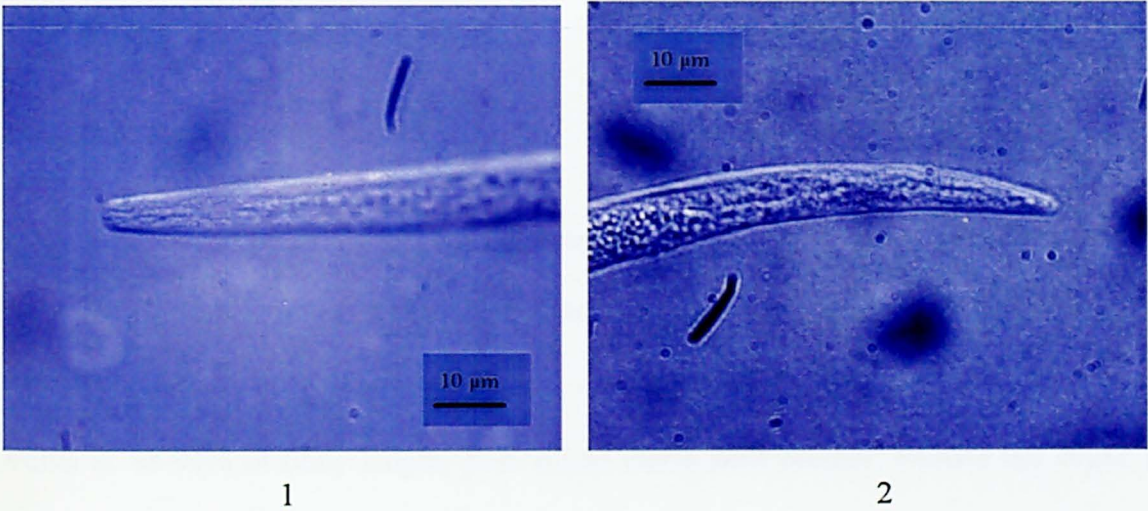
Tylenchida spp. occur in various possible habitats such as soil, water and plants. Most of them live in soil and feed on plant roots. Early *Tylenchida* in phylogenetic relationship must have been bulk feeders on bacteria, fungi and algae with their stylet-like stoma. Later, they evolved as fungal feeders then as plant parasites (Siddiqi, 2000).

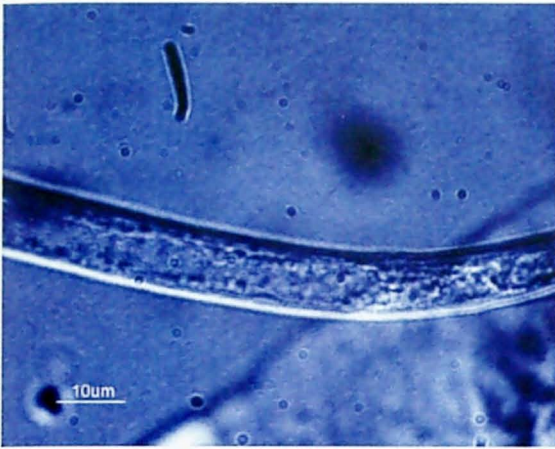
In this study, the *Tylenchida* sp. 1 was extracted more frequently from wood pieces than from bark. This nematode built up its population density from wood pieces placed on PDA. For these reasons, this nematode was regarded as fungal feeder vectored by some beetle, though more studies are needed to clarify the bionomics of this species.

GENERAL CHARACTERS

Order Tylenchida is generally characterized by mostly lateral position of aphid. Stylet shaft mostly formed by metarhabdions. Median oesophageal bulb, if present, without muscular valve. Anus inconspicuous, minute, pore-like, directed outward. Sperm usually small size. Male caudal papillae absent, bursa lacking papillary ribs or rays and never present only at the tail tip. Spicule not thorn shape (Siddiqi, 2000).

Unfortunately, I could not get detailed information of the morphological characters of this species due to a few number of specimen available for observation. The species has slender body of medium to long length. Cephalic region low, smooth.

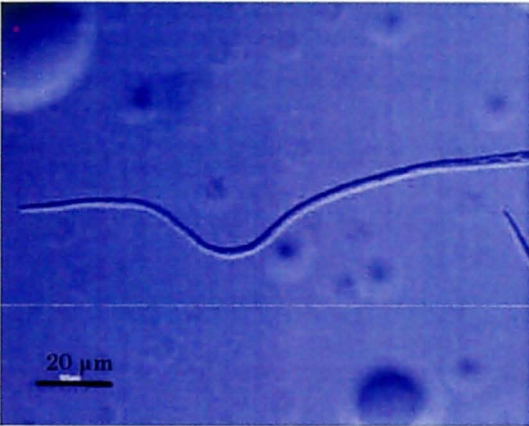




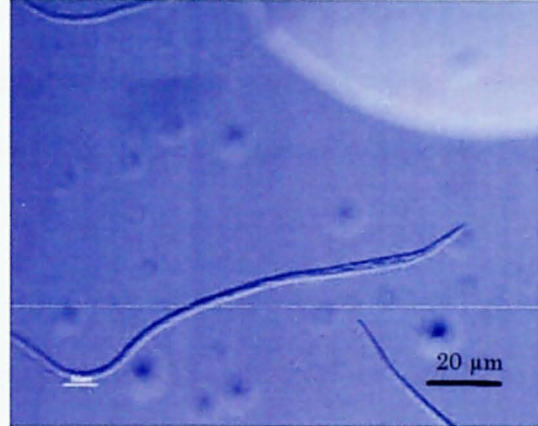
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Fig. 5-N. Tylenchida sp. 1.

1. Female head; 2. Female tail; 3. Vulva; 4. Male tail; 5-6. Adult female.

Vulva at 88-94% of body length, post vulval uterin sacch present. Female tail slightly conoid but with rounded tip (Fig. 5-N).

15. Tylenchida sp. 2

BIONOMICS

This Tylenchida species share common properties in the bionomics with the

previous Tylenchida sp. 1. Especially in superfamily Anguinoidea, some of the nematodes are usually fungal feeders and parasites of aerial parts of plant (Siddiqi, 2000).

GENERAL CHARACTERS

Stylet shaft mostly formed by metarhabdions, median oesophageal bulb, if present, without muscular valve. Anus inconspicuous, sperm usually small size. This species has typical characteristics of Tylenchida in present of bursa in ventrally spicule.

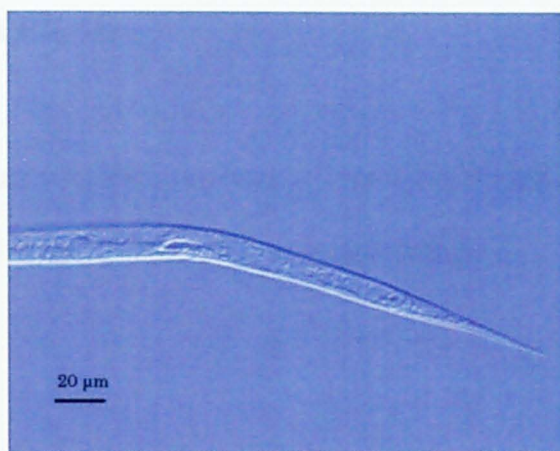
The Tylenchida sp. 2 has several characters of the superfamily Anguinoidea: female tail conoid to filiform, rarely subcylendroid, which is similar to that of male; bursa not enveloping tail terminus.

The characters found in this species are similar to those of genus *Nothotylenchus*; the species has cephalic region frame in six sectors. Spear with rounded basal knobs. Corpus and esophagus cylendroid, with or without fusiform valveless bulb. Posterior uterin branch present. Spicule and gubernaculum tylenchoid. Bursa peloderan. Based on these characters, this species might be a species of the genus *Nothotylenchus* in infraorder Anguinina

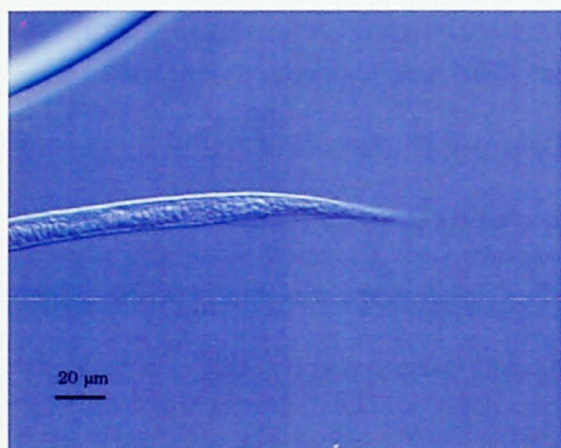
Tylenchida sp. 2 and Tylenchida sp. 1 can be separated by the character of their tail tips. Female tail tip of the Tylenchida sp. 1 is slightly conoid and rounded, while female of Tylenchida sp. 2 has tapered tail with rounded tip, elongated, post uterin-sach present (Fig. 5-O). Further studies are needed to identify and describe these two species habiting in dead pine trees.



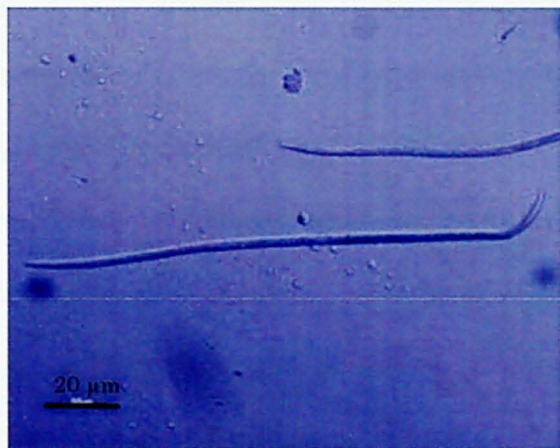
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Fig. 5-O. *Tylenchida* sp. 2.

1. Female head; 2. Female tail with vulva; 3. Male tail; 4. Adult female.

CHAPTER III

Cohabitation of the pinewood nematode, *Bursaphelenchus xylophilus*, and fungal species in pine trees inoculated with the nematode.

Introduction

Some researchers (Kobayashi *et al.*, 1974, 1975; Maehara and Futai, 2000; Wang *et al.*, 2005) have indicated that PWN does not multiply on sterilized pinewood in the absence of fungi. Among the fungal species isolated from a wilt-killed pine tree, some fungi such as *Ceratocystis* sp., *Diplodia* sp. and *Pestalotia* sp. are reported to be suitable foods, while others are unsuitable (Kobayashi *et al.*, 1974, 1975; Fukushige, 1991). Wang *et al.* (2005) demonstrated that the number of nematodes propagated in *Pinus thunbergii* cuttings was high at sites where fungal hyphae distributed. Under laboratory conditions, Maehara and Futai (1996, 1997) demonstrated that fungal species affected not only PWN multiplication but also the number of PWNs carried by a vector beetle. These findings clearly indicate that fungal flora in a dead pine tree might be one of the most determinative biotic factors for PWN multiplication and distribution in the tree.

The influence of fungi on the multiplication of PWN has been well investigated by many researchers under laboratory conditions as mentioned above. However, the influence on distribution of PWNs in host trees has not been so well understood especially under natural conditions, in which many factors could influence on the population density of PWNs as well as the fungal flora itself. Hence, field studies with intensive sampling are needed.

In the present study, I inoculated 15-year-old *P. thunbergii* trees grown outdoors with the PWN, investigated seasonal change of the fungal flora in the trees and analyzed the effect of fungal flora on the distribution of PWN at the microhabitat level. I also examined PWN propagation on fungi isolated from the field in the following chapter. On the basis of these two experiments, the effect of each fungal species on the distribution and propagation of PWN are discussed.

Materials and Methods

EXPERIMENTAL TREES SERVED FOR THE INVESTIGATION OF SEASONAL CHANGES IN FUNGAL FLORA

Pine trees used for the investigation of fungal flora were the same trees that served for the investigation of seasonal changes in nematode fauna, and so were inoculated with PWNs and then received *Monochamus alternatus* larvae into their stems. Detailed information was described in Chapter I.

WOOD SAMPLING

On 10 August, 13 October and 16 December 2004 and 9 February, 10 April and 10 June 2005, three trees were cut down and a 10-cm-long bole wood block was arbitrarily collected from each tree containing one of the *M. alternatus* larva-introduced points where a pupal chamber (PC) had been made. Each of the bole blocks was sliced into eight 1-cm thick discs, and 2 x 2 cm lattices were drawn on the cut surfaces. Then

the discs were photocopied to record the position of PCs and tunnels of *M. alternatus*, and each of the discs was cut into small pieces (2 x 2 x 1 cm) along the line of the lattice. Each piece of wood thus prepared was split into two halves (2 x 1 x 1 cm). One-half served nematode isolation to investigate seasonal changes in nematode fauna and the other half was stored at 4°C until fungal isolation.

FUNGAL ISOLATION

Within two days after collection of wood samples, the remaining half-cut wood pieces (2 x 1 x 1 cm) kept at 4°C were surface sterilized for 1 second on the flame of burner before placing them on potato dextrose agar (PDA) in a Petri dish for fungal isolation. After 3-day incubation at 20°C, various fungi with different appearance grew on the plate. A small piece of agar with fungal mycelium was taken from each of the colonies with different appearance grown on the plates. They were put onto malt extract agar with chloramphenicol (2.0% malt extract, 1.5% agar, 100-ppm chloramphenicol) to establish pure cultures. This procedure was repeated every other month, after collection of samples. Isolation of fungi from healthy trees was done on June 2005 following the same procedure as mentioned above.

FUNGAL IDENTIFICATION

Fungal mycelia were picked up by randomly scrapping the surface with a scalpel from the fungal cultures, bimonthly. A chemical procedure was used to homogenize the fungal body; the fungal cell wall was first digested by adding 50 µl of 1% Westase

(TaKaRa) solution and incubating at 30°C for two hours. The digested solution was then transferred into a new 1.5 ml tube containing 100 µl of CTAB solution and DNA was extracted and purified according to Matsuda and Hijii (1999). The internal transcribed spacer (ITS) region of the ribosomal DNA was amplified using primers ITS1-F (5'-CTT GGT CAT TTA GAG GAA GTA A-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3'; Gardes and Bruns, 1993; White *et al.*, 1990). The product was digested with two restriction enzymes, *Alu-I* and *Hinf-I*. The fungal samples were divided into genotypes according to their PCR-RFLP patterns. When PCR-RFLP patterns of one sample did not match those of another, the two were considered to belong to different genotypes. Frequent genotypes, those detected from more than 50% of the bimonthly samples, were sequenced according to White *et al.* (1990) for ITS1, ITS2 and 5.8S regions of ribosomal DNA, using the primer ITS1F and ITS4. DNA sequences were determined using a genetic analyzer (3130, Genetic Analyzer, Applied Biosystems, USA). The DNA sequence determined for each fungus was compared with that of known species in the GenBank database. Identification at the genus level was based on identities above 90%. I also confirmed morphologically by sending the pure culture to a mycologist at Forestry and Forest Products Research Institute, Japan.

Representative cultures are maintained in the Mycological Collection of the Laboratory of Environmental Mycoscience, Graduate School of Agriculture, Kyoto University, Kyoto, Japan.

DETECTION FREQUENCY OF FUNGAL SPECIES ISOLATED

For the investigation of seasonal changes in fungal flora, the presence of each fungal species was determined by observing the morphology and color of fungal colonies grown on the PDA plate where the wood samples were placed. If a designated fungal species had been detected from a half-cut wood piece, the sample was classified as 'positive', otherwise 'negative'. To calculate detection frequency of fungal isolates, the number of positive wood pieces was divided by the total number of wood pieces harvested from each trees.

ANALYSIS OF COMPATIBILITY BETWEEN EACH FUNGAL SPECIES AND THE PWN

To calculate the average PWN number over fungal positive pieces harvested from each tree, sum of the number of PWN extracted from them was divided by sum of their dry weight. Likewise, a harmonic average of PWN number over fungal negative wood pieces was calculated. Here I denote the two averages as N_f and N_0 , respectively. I estimated the influence of each fungus on the population growth and settlement of PWN using 'Nematode population ratio (NPR)', which is defined as the ratio of N_f to N_0 . This value shows relative abundance of PWN yielded on the samples with a given fungal species, and becomes greater than one when a given fungus facilitates the growth or settlement of PWN population, on the contrary it becomes less than one when a given fungus suppress the growth or settlement of PWN population.

I also calculated 'Index of cohabitation ability (ICA)' focusing on the relationship between the presence of a given fungi and that of the PWN as follows;

$$ICA = \text{Log} \frac{(A_n + 0.01) \times (B_0 + 0.01)}{(A_0 + 0.01) \times (B_n + 0.01)},$$

where;

A₀ = Total number of wood pieces which contained only the fungus per tree,

A_n = Total number of wood pieces which contained both fungus and nematode per tree,

B₀ = Total number of wood pieces which contained neither fungus nor nematode per tree,

B_n = Total number of wood pieces which contained only nematode per tree.

This index shows the degree of co-occurrence of PWN with a given fungus, positive ICA indicating a tendency of co-occurrence, while negative one repulsion.

Results

SEASONAL CHANGES OF THE PINWOOD NEMATODE POPULATION IN STANDING PINE TREES

As shown in Fig. 6, the number of nematodes was high in August, but decreased slightly in December then recovered in February, and finally decreased again until the end of the experiment (June), though the data varied among every tree individuals except for those of August.

IDENTIFICATION OF THE FUNGI AND THEIR DETECTION RATE

Eighteen species of fungi in total were identified to the genus level. The results were confirmed morphologically by Dr. Kubono, a mycologist, Forestry and Forest Products Research Institute, Japan. Possible species, which had the highest nucleotide

identity with the fungal genotype detected, are *Aspergillus* sp., *Aureobasidium* sp., *Fusarium* sp. 1, *Fusarium* sp. 2, *Gliocladium* sp., *Mucor* sp., *Mortierella* sp., *Penicillium* sp. 1, *Penicillium* sp. 2, *Penicillium* sp. 3, *Pestalotiopsis* sp. 1, *Pestalotiopsis* sp. 2, *Phialophora repens* (R.W. Davidson) Conant., *Rhizoctonia* sp., *Sphaeropsis sapinea* (Fr.) Dyko & Sutton., *Trichoderma* sp. 1, *Trichoderma* sp. 2 and *Trichoderma* sp. 3. Fungal identification is summarized in Table 2. The mycelia of dominant fungal growth on PDA were shown in Fig. 7.

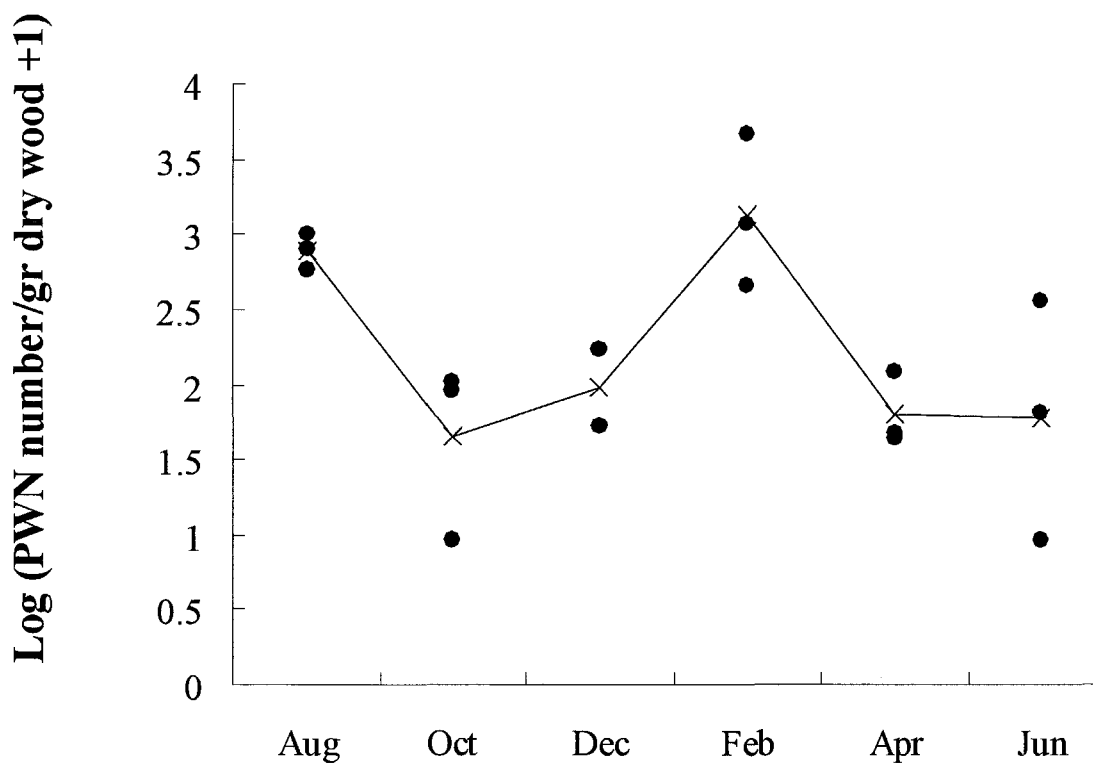


Fig. 6. Seasonal changes of PWN on dead pine trees (n=3). A closed circle indicates a log-transformed average nematode density in a pine tree. To calculate the average, the total number of PWN extracted from the samples harvested from each tree was divided by their total dry weight. A cross indicates a log-transformed arithmetic average of the three average nematode densities for each sampling time.

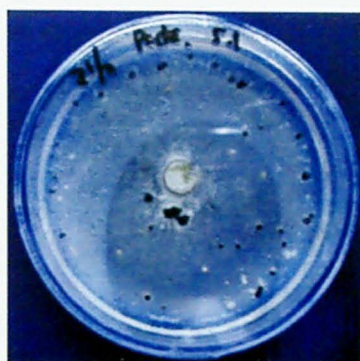
Table 2. Fungi isolated from dead pine trees inoculated with PWN and healthy trees

Isolated from dead pine trees	Isolated from healthy pine trees
<i>Aspergillus</i> sp.	<i>Aspergillus</i> sp.
<i>Aureobasidium</i> sp.	<i>Gliocladium</i> sp.
<i>Fusarium</i> sp. 1	<i>Mucor</i> sp.
<i>Fusarium</i> sp. 2	<i>Penicillium</i> sp. 1
<i>Gliocladium</i> sp.	<i>Penicillium</i> sp. 2
<i>Mucor</i> sp.	<i>Penicillium</i> sp. 3
<i>Mortierella</i> sp.	<i>Pestalotiopsis</i> sp. 1
<i>Penicillium</i> sp. 1	<i>Pestalotiopsis</i> sp. 2
<i>Penicillium</i> sp. 2	<i>Phialophora repens</i>
<i>Penicillium</i> sp. 3	<i>Sphaeropsis sapinea</i>
<i>Pestalotiopsis</i> sp. 1	<i>Trichoderma</i> sp. 1
<i>Pestalotiopsis</i> sp. 2	<i>Trichoderma</i> sp. 2
<i>Phialophora repens</i>	<i>Trichoderma</i> sp. 3
<i>Rhizoctonia</i> sp.	
<i>Sphaeropsis sapinea</i>	
<i>Trichoderma</i> sp. 1	
<i>Trichoderma</i> sp. 2	
<i>Trichoderma</i> sp. 3	

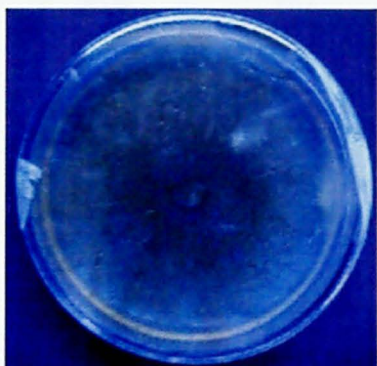
Fungal floras in the trees inoculated with PWN under natural conditions were different from tree to tree. Among the 18 fungal species identified, *P. repens*, *S. sapinea*, two *Pestalotiopsis* spp. and *Rhizoctonia* sp. were detected most frequently every season (Fig. 8). It was impossible to distinguish between two *Pestalotiopsis* species from morphological observation, so I treated these two species as *Pestalotiopsis* spp. I focused on these four frequent species and examined them in more detail. *Penicillium* sp. and *Trichoderma* sp. were also examined for comparison.



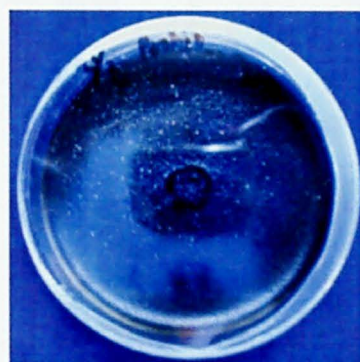
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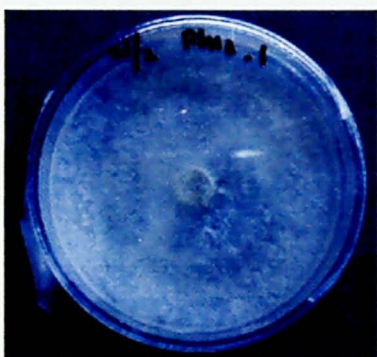
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Fig. 7. The mycelia of dominant fungal growth on PDA

1. *Pestalotiopsis* sp. 1; 2. *Pestalotiopsis* sp. 2; 3. *Phialophora repens*; 4. *Sphaeropsis sapinea*; 5. *Rhizoctonia* sp.

Detection frequency of fungi isolated from inoculated trees

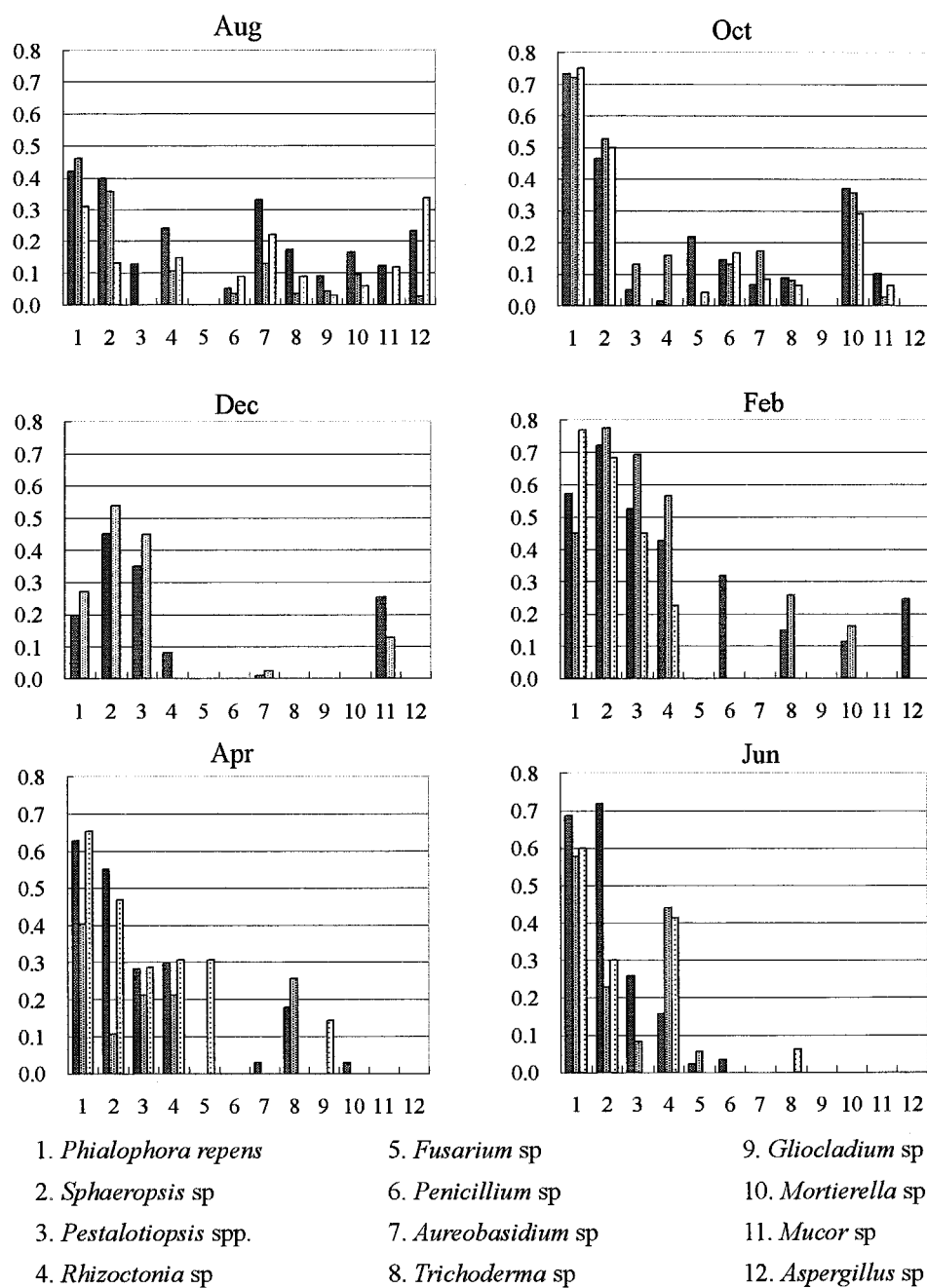


Fig. 8. Seasonal changes in frequency of fungi isolated from inoculated trees. * Ratio of the numbers of fungal positive wood pieces to the total number of wood pieces harvested from each trees

INFLUENCE OF EACH FUNGUS ON THE PRESENCE AND/OR PROPAGATION OF PWN

To evaluate the influence of each fungus on the presence and/or propagation of PWN, the NPR was calculated (Fig. 9). NPR of four dominant fungal species were slightly higher than one every season, suggesting these fungi facilitate the population growth of PWN. In contrast the NPR of *Trichoderma* sp. and *Penicillium* sp. were lower than and equal to one, respectively. *Trichoderma* sp. suppressed the population growth of PWN, and *Penicillium* sp. influenced neither positively nor negatively on the population growth. Thus, these nematodes preferably aggregated to dominant fungi over the experimental period, but promotive effects of each fungus on the presence and/or propagation of PWN were changed from season to season and from tree to tree. The other less abundant fungal species were not analyzed in detail.

COHABITATION OF PWN AND FUNGAL SPECIES IN PWN-INOCULATED TREES

The ICA values which focused only on the presence or absence of fungi and the PWN were slightly higher than zero for three dominant fungal species, *P. repens*, *S. sapinea*, *Pestalotiopsis* spp., while those for *Penicillium* and *Rhizoctonia* species were around zero, and that for *Trichoderma* sp. was slightly lower than zero (Fig. 10). Thus, three dominant fungi tended to cohabit with PWN, while *Trichoderma* sp. showed repelling effects towards PWN. Both *Penicillium* sp. and *Rhizoctonia* sp. had no special relationship with PWN regarding their distribution.

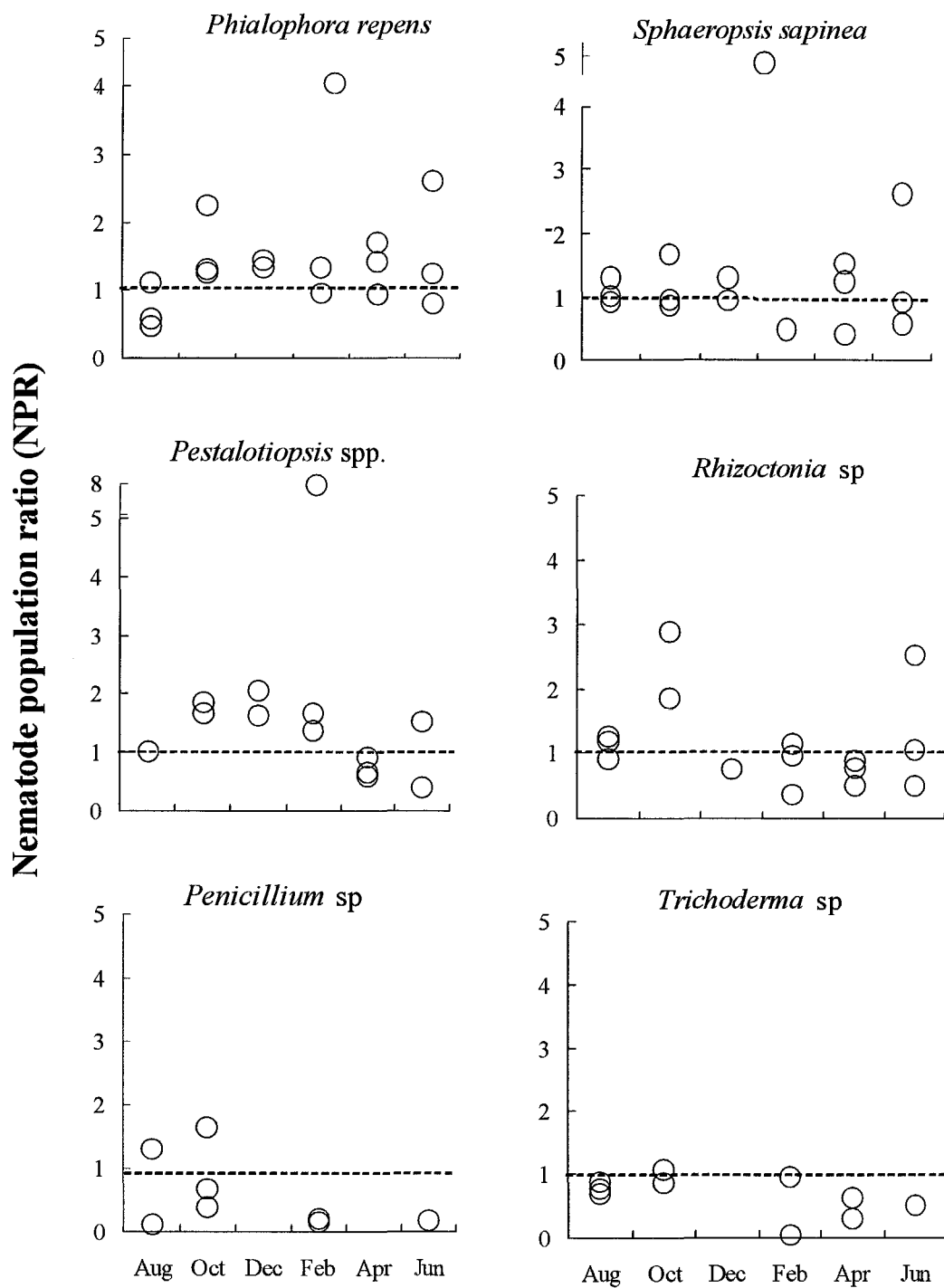


Fig. 9. The effect of each fungal species on the distribution of PWN in dead pine trees. * Ratio of Nf to N0, where Nf and N0 are average PWN numbers over fungal positive and negative wood pieces harvested from each pine tree, respectively.

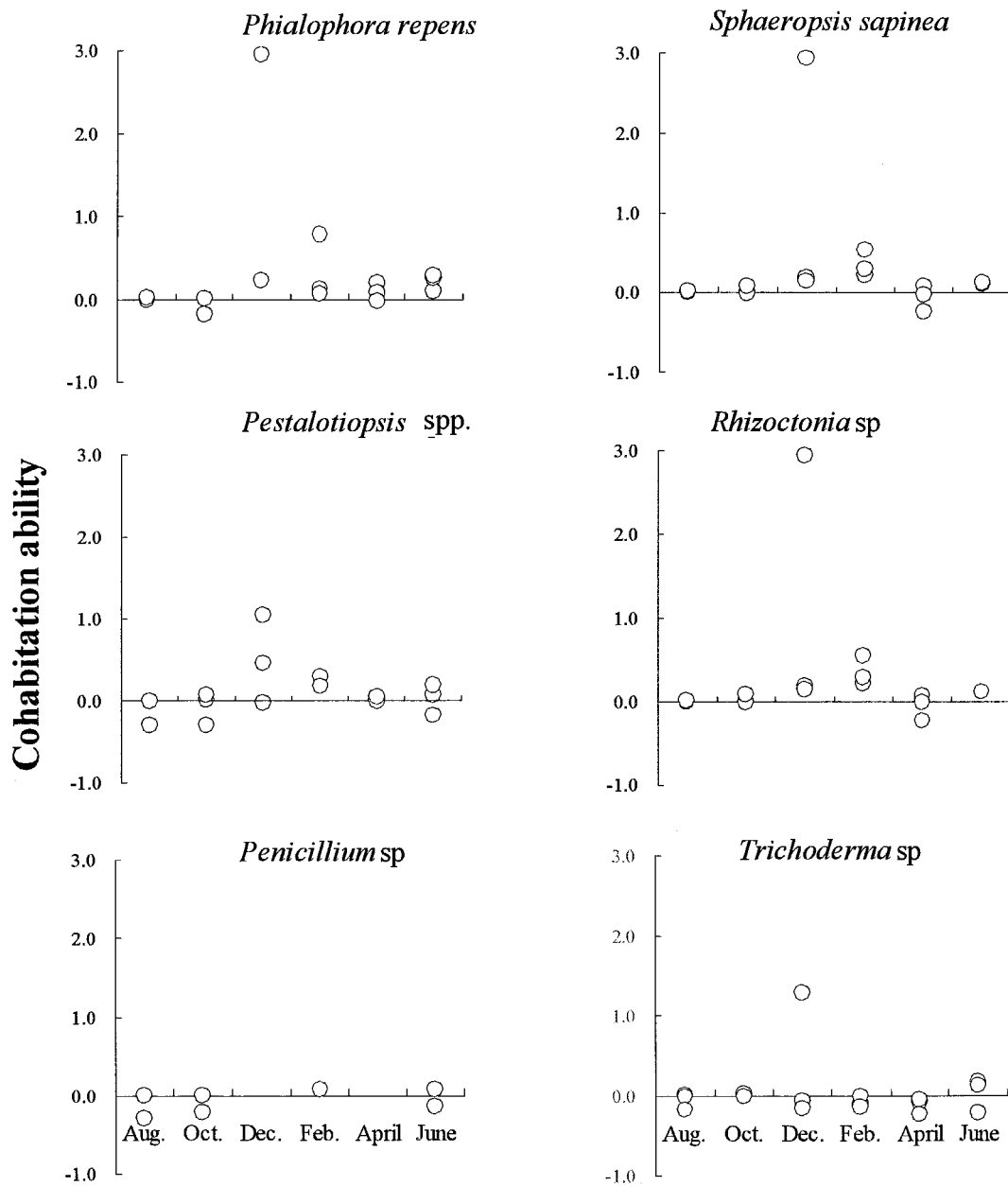


Fig. 10. Cohabitation ability between PWN and each fungal species. * ICA = $\text{Log} \left(\frac{(A_n + 0.01) \times (B_0 + 0.01)}{(A_0 + 0.01) \times (B_n + 0.01)} \right)$, where A_0 is the total number of wood pieces which contained only the fungus per tree; A_n is the total number of wood pieces which contained both fungus and nematode per tree; B_0 is the total number of wood pieces which contained neither fungus nor nematode per tree; B_n is the total number of wood pieces which contained only nematode per tree.

Discussion

From the wood samples of the pine trees examined, 18 fungal species were isolated. Among them, *P. repens*, *S. sapinea*, two *Pestalotiopsis* spp. and *Rhizoctonia* sp. were frequently isolated and were considered as dominant fungi. Although the dominant fungi were constantly detected over the experimental period, the composition of fungal species slightly varied among seasons as reported in previous studies (Kobayashi *et al.*, 1974; 1975; Fukushige and Futai, 1987; Kuroda and Ito, 1992). For example, Kuroda and Ito (1992) reported that the fungal species detected from PWN-inoculated pines were the same with those in healthy trees during 4 weeks after inoculation. The species detected both from healthy and inoculated pine trees were *Pestalotiopsis* spp., *Nigrospora* spp., *Cladosporium* spp. and *Phomopsis* spp. They found a blue stain fungus, *Ceratocystis* sp., and bacteria 5 weeks after nematode inoculation. In this study, minor fungi disappeared when pine trees were completely killed in December, and fungal flora of the pine trees gradually changed until June, the end of this experiment.

Kobayashi *et al.* (1974) recorded one species of *Diplodia* as one of the common fungi in the wood of dead pine trees affected by *B. ligniculus* (previous name of *B. xylophilus*). They also revealed that the *B. ligniculus* multiplied well on its mycelia grown on PDA plate medium. In the present study, I frequently isolated *S. sapinea*, which is regarded as a synonym of *Diplodia* (Denman *et al.*, 2000). De Wet *et al.* (2003) also suggested that *S. sapinea* should be reverted to the former name of *Diplodia pinea*. To identify the fungi detected from PWN-inoculated pine trees, I, however did not follow the system of Dennann *et al* (2000), because I depended on the GenBank data, in which the identical sequence to my data was not provided under *D. pinea* but *S.*

sapinea.

A close affinity between the PWN and the blue-stain fungi has been reported in several studies (Kobayashi *et al.*, 1974; 1975; Fukushige, 1991; Maehara and Futai, 2000; Maehara *et al.*, 2005). Kobayashi *et al.* (1974, 1975) considered that *Ophiostoma* species might be transmitted by *Monochamus* beetle when beetles feed on young shoot of healthy pine trees in early summer. In the present study, however, this group of fungal species was not isolated. This may be attributed to the fact that the pine trees had been killed by artificial inoculation with the PWN and thereby microbial environments might have become unsuitable for *Ophiostoma* even when it was transmitted by *Monochamus* beetle.

To evaluate the effect of fungal species and their distribution on the population density and distribution of PWN, two indices, NPR and ICA, were calculated. NPR compares the relative abundance of PWN between samples with and without a designated fungal species. ICA reflects degree of co-occurrence of a fungus and PWN. In the case of the dominant fungi except for *Rhizoctonia* sp., NPR was higher than one and ICA was higher than zero, which means that PWN had a tendency to coexist with these fungi and/or propagate well on them. In the case of the other minor fungi, e.g., *Trichoderma* spp., NPR and ICA were less than one and zero, respectively, showing an incompatible relationship to the PWN. Thus, these facts suggest that the dominant fungi in pine trees killed by PWN promoted the propagation and/or the settlement of the nematode.

CHAPTER IV

Propagation of the pinewood nematode, *Bursaphelenchus xylophilus*, on eighteen fungal species isolated from pine trees killed by the nematode.

Introduction

The suitability of fungi for the propagation of PWN has been well investigated by many researchers under laboratory conditions. For instance, Kobayashi *et al.* (1974, 1975) and Fukushige (1991) compared the propagation of PWN on the mycelial colonies of *Trichoderma* spp., *Chepalosporium* sp., *Penicillium* sp. and *Verticillium* sp. fungi isolated both from healthy and dead pine trees. They revealed that PWNs fed and propagated well on some of the fungi isolated from dead pine trees, while they neither fed nor propagated on some other fungi isolated from dead pine trees.

In the previous chapter, I examined the relationship between the PWN and the fungi cohabiting in dead pine trees. In the present study, most of the dominant fungi in pine trees, such as *Sphaeropsis sapinea*, *Phialophora repens* and two *Pestalotiopsis* spp., showed a compatible relationship with PWN, i.e., these fungal species showed a pronounced tendency to coexist with PWN. The dominant fungi seemed to promote PWN propagation as suitable food, which might be one of the reasons why PWN has a high cohabiting tendency with these fungi. In the case of the other fungi, their cohabiting tendency was lower. This might be due to their unsuitability as food of PWN

and/or due to the repellent they might produce. These speculations based on the results from the field experiment should be confirmed under laboratory conditions. In the present chapter, therefore, I examined PWN propagation on 18 fungal species isolated from dead or dying pine trees.

Materials and Methods

The following 18 fungal species isolated from dead or dying pine trees served as food source of PWN; *Aspergillus* sp., *Aureobasidium* sp., *Fusarium* sp. 1, *Fusarium* sp. 2, *Gliocladium* sp., *Mucor* sp., *Mortierella* sp., *Penicillium* sp. 1, *Penicillium* sp. 2, *Penicillium* sp. 3, *Pestalotiopsis* sp. 1, *Pestalotiopsis* sp. 2 and *P. repens*, *Rhizoctonia* sp., *S. sapinea*, *Trichoderma* sp. 1, *Trichoderma* sp. 2, *Trichoderma* sp. 3. *Botrytis cinerea*, the gray mold of many fruits and vegetables was used as control. Each of these fungi was transferred onto a 6 cm diam. Petri dish containing 5 ml of PDA, and then incubated at 20°C. When the diameter of the colony reached approximately 5 cm, 300 PWNs were inoculated onto each fungal colony. Five, 10 and 15 days after PWN inoculation, nematodes were extracted from the plates of each of the fungi by the Baermann funnels, and counted.

Result

There was a significant difference in the total number of PWN per plate among 19 fungal species examined ($p < 0.01$) and among sampling times ($p < 0.01$ by Tukey Kramer's test; Table 3). The largest population of PWN was recorded on *S. sapinea*, though it was not significantly different from that on *B. cinerea*, the control fungus. The

nematode population increased markedly from 5 to 10 and from 10 to 15 days after inoculation on *S. sapinea*. On the other dominant fungi in dead pine trees, such as two species of *Pestalotiopsis* and *P. repens*, population density of PWNs at 15 days after inoculation were significantly lower than those on *B. cinerea* or on *S. sapinea*. On two of the three species of *Penicillium*, one of the two species of *Fusarium* and on a species of *Aureobasidium*, population density of PWNs slightly increased from 5 to 10 days after inoculation, and then decreased until 15th days. The populations density of PWN built up on 19 fungal species were compared in Table 3.

Discussion

In Chapter 3, I reported 18 fungal species inhabiting in dead pine trees killed by PWN. Among the 18 fungal species identified, *P. repens*, *S. sapinea*, two *Pestalotiopsis* spp., and *Rhizoctonia* sp. were detected most frequently every season. The former four dominant fungi were supposed to facilitate the population growth of PWN (NPR was slightly higher than unity). These dominant fungi also showed a tendency to cohabit with PWN (ICA was higher than zero). The dominant fungi seemed to serve PWN as suitable food thereby facilitate its population growth, which might be one of the reasons why PWN has a high cohabiting tendency with these fungi.

This speculation from the field experiment was confirmed by the present laboratory experiment. PWN propagated well on the colonies of the dominant fungi except for *Rhizoctonia* spp., detected from PWN-killed pine trees, i.e., *S. sapinea*, *P. repens* and two *Pestalotiopsis* spp., grown on PDA plate medium. Especially, PWN multiplied as well on *S. sapinea* as on *B. cinerea*, which is commonly used to rear PWN

under laboratory conditions. On the other hand, no or little propagation was observed on the colonies of three species of *Trichoderma*, two species of *Fusarium*, three species of

Table 3. Population density of PWN built up on 19 fungal species.

Fungi tested	Population density (Log. no. of nematode +1)					
	5 days		10 days (after inoculation)		15 days	
<i>Aspergillus</i>	0.000±0.000	a	0.000±0.000	a	0.000±0.000	a
<i>Gliocladium</i> sp.	0.202±0.096	a	0.000±0.000	a	0.000±0.000	a
<i>Mortierella</i> sp.	0.000±0.000	a	0.000±0.000	a	0.000±0.000	a
<i>Mucor</i> sp.	0.000±0.000	a	0.000±0.000	a	0.000±0.000	a
<i>Penicillium</i> sp.1	1.604±0.071	bcdefg	1.270±0.111	b	0.000±0.000	a
<i>Trichoderma</i> sp.1	0.000±0.000	a	0.000±0.000	a	0.000±0.000	a
<i>Trichoderma</i> sp.2	0.000±0.000	a	0.000±0.000	a	0.000±0.000	a
<i>Trichoderma</i> sp.3	0.000±0.000	a	0.000±0.000	a	0.000±0.000	a
<i>Fusarium</i> sp.2	0.804±0.091		1.544±0.086	b	0.228±0.160	a
<i>Penicillium</i> sp.2	1.484±0.047	bcdef	1.952±0.069	cde	1.587±0.073	bc
<i>Penicillium</i> sp.3	1.744±0.072	cdefghi	2.173±0.021	de	1.861±0.021	bcd
<i>Aureobasidium</i> sp.	1.658±0.062	bcdefgh	1.833±0.070	cd	1.889±0.018	cde
<i>Rhizoctonia</i> sp.	1.371±0.090	bcde	2.392±0.095		2.172±0.048	de
<i>Fusarium</i> sp. 1	1.349±0.077	bcde	2.981±0.072	f	2.485±0.091	
<i>Phialophora repens</i>	1.838±0.083	defghi	3.081±0.027	f	5.135±0.073	fg
<i>Pestalotiopsis</i> sp.1	2.055±0.050	fghij	3.48±0.040		5.421±0.072	fgh
<i>Pestalotiopsis</i> sp.2	2.226±0.065	hij	3.828±0.036	g	5.65±0.070	gh
<i>Sphaeropsis sapinea</i>	1.832±0.116	defghi	4.022±0.106	g	6.156±0.036	I
<i>Botritis cinerea</i>	1.945±0.016	efghij	3.785±0.018	g	6.23±0.018	I

Means followed by the same letters in a column are not significantly different at 5% level (Tukey-Kramer's test)

Penicillium, and each species of *Mucor*, *Mortierella*, *Gliocladium*, *Aspergillus*, *Rhizoctonia* and *Aureobasidium*. Some researchers also reported that some species of

Trichoderma and *Penicillium* were unsuitable for nematode propagation (Kobayashi *et al.*, 1974, 1975; Maehara *et al.*, 2000). Changes of PWN population density with passage of time were examined on *B. cinerea* by Ishibashi and Kondo (1977), on species of blue stain fungus *Ophiostoma picea* (Munch) Syd. & P. Syd. by Forge and Sutherland (1996); resulting that each of these fungi was suitable for PWN reproduction. Blue stain fungus, *O. minus* (Hedgc.) Syd.& P. Syd. is the most common fungus that was isolated from dead pine trees and proved to be an important factor in determining the distribution of PWNs (Maehara *et al.*, 2005). In the present study four dominant fungi detected from PWN-killed pine trees were also proved to be good food source of PWN, though any *Ophiostoma* species could not be detected from such pine trees. This means that there are suitable fungi for PWN reproduction besides blue stain fungi in wilt-killed pines. It is thought, therefore, that PWN can survive and propagate well in dead pine trees feeding on these dominant fungi.

GENERAL DISCUSSION

From practical point of view, the population dynamics of PWNs in dead pine trees has been one of our major concerns, because the density of PWNs in dead pine tree seems to influence on the number of PWNs carried by a vector beetle, *Monochamus alternatus*. In addition, the number may determine the ability of each beetle in spreading the pine wilt disease. From the biological viewpoint, however, the population changes of PWNs could be regarded as a part of sequential microbial relationships occurring in the process of wood degradation.

The present study clarified under natural conditions, i) how nematode fauna and fungal flora in dead pine trees changed after infection with PWN, and ii) how dominant nematode species and dominant fungal species influenced on the population density of PWNs by analyzing their population ratio (NPR) and cohabiting ability (ICA).

In Chapter I, fifteen species of nematodes were identified, including five mycophagous species, nine saprophagous species and one predatory species (Fig. 1). Three species of *Bursaphelenchus* including PWN, *Bursaphelenchus xylophilus* were detected. One species of them was identified as new species (Fig. 5-B). The number of the PWNs decreased from October to December, increased in February, then, it decreased again until June when the experiment terminated, as is similar fluctuation in the number of *B. xylophilus* reported by Fukushige and Futai (1987). This fluctuation in the population density of *B. xylophilus* seemed to synchronize with that of *Diplogasterida* sp. 1 on each tree throughout the experimental period while any other free-living species did not show such synchronization (Fig. 2). Based on its morphological characters (Chapter II) *Diplogasteridae* sp. 1 seems to belong to the

genus *Rhabdontolaimus* (Fig. 5-D). Kanzaki *et al.* (2002) found both a species of *Bursaphelenchus* and a species of *Rhabdontolaimus* in their vector beetle's body and in their host fig trees. They described these nematodes as new species, *Rhabdontolaimus psacothaeae* and *Bursaphelenchus conicaudatus* and supposed them to be sympatric. Differences in their food preference in host trees and in the part of the vector body must enable *R. psacothaeae* and *B. conicaudatus* to be sympatric. A large number of *Diplogasteridae* sp. 1 also supported that result and both *Diplogasteridae* sp. 1 and PWN were congregated around both PCs and tunnels in dead pine trees, perhaps because feeding habit of these two nematodes might be different. Other free-living nematodes including saprophagous and mycophagous species were detected in small population. The factors that affect the population dynamics of nematode fauna are still unclear, thought food source of each nematode species should be one of the important factors. As Shigo (1967) stated, when trees become diseased the physical conditions within the trees, such as water content, temperature, and so on must change dramatically. The transition of season should also affect environmental conditions. This is the reason why this study was conducted bimonthly, and each sampling time was under different climate conditions, however, there was no correlation between nematode numbers and host water content (Fig. 4).

To determine the effect of the population density of *Diplogasterida* sp. 1 on that of PWN population, the correlation between them was analyzed at the microhabitat levels, although consistent correlation patterns were rarely observed (Fig. 2). This might be attributed to the limited volumes of sample tissues: the distributions of either nematode among disks of wood pieces (2 x 1 x 1cm) were more or less at random. PCs and tunnels of vector beetles were also another important factor causing the difference in the

population dynamics of PWN under natural conditions. Among 15 species of nematode detected, only mycophagous *B. xylophilus* and saprophagous Diplogasterida sp. 1 were more abundant around PCs and tunnels than elsewhere in the wood (Fig. 3). PCs and tunnels might provide suitable humidity and sufficient nutrients for growth and reproduction of fungi and other microbes, and therefore for those of nematodes feeding on such microbes. Kobayashi *et al.* (1974, 1975), Fukushige (1991) and Maehara and Futai (2000) reported that the blue-stain fungi which prevailed around PCs would serve as food for *B. xylophilus*.

Among 18 fungal species that were isolated from dead pine trees infested with PWN, *Phialophora repens*, *Sphaeropsis sapinea*, two *Pestalotiopsis* spp. and *Rhizoctonia* sp. were frequently isolated and were considered as dominant fungi (Fig. 8). Although the dominant fungi were constantly detected over the experimental period, the composition of fungal species slightly varied among seasons as reported in previous studies (Kobayashi *et al.*, 1974; 1975; Fukushige and Futai, 1987; Kuroda and Ito, 1992). At a beginning of winter, minor fungi had disappeared when pine trees were completely killed, and fungal flora of the pine trees gradually changed until the several fungi remained as dominant fungi at the end of this experiment.

Blue stain-fungi, *Ophiostoma* spp. are the main food of PWN in dead pine trees (Fukushige, 1991) and there have been some reports on the close relation between PWN and blue-stain fungi (Kobayashi *et al.*, 1974; 1975; Fukushige, 1991; Maehara and Futai, 2000; Maehara *et al.*, 2005). However, this group of fungal species was not isolated throughout this experiment.

Two indices, Nematode population ratio (NPR) and Index cohabitating ability (ICA) were calculated to evaluate the effect of fungal species on the population density

and distribution of PWN. PWN had a tendency to coexist with the dominant fungi and/or propagate well on them, although the minor fungi, e.g., *Trichoderma* spp., showed an incompatible relationship to the PWN (Fig. 9 and 10). The dominant fungi promoted PWN propagation as suitable food, which might be one of the reasons why PWN has a high cohabiting tendency with these fungi. In the case of the other fungi, their cohabiting tendency was lower. This might be due to the feature of those fungi to suppress PWN propagation and/or the repellent they might produce. These results strongly suggest that dominant fungi in pine trees killed by PWN served as good food for PWN propagation. The dominant fungi were detected also in healthy pine trees (Table 2) and might be neither pathogenic nor parasitic fungi inhabiting pine trees. Thus not only blue stain fungi but also other fungal flora should determine the distribution and population change of PWNs in the natural field, as various fungal species besides blue stain fungi had been found as suitable food for PWN by laboratory experiment.

This study focused on the relationship between the PWN and nematode fauna and fungi cohabiting in dead pine trees, and examined the role of each fungus from two points of view; suitability of fungi as food of PWN and feature of fungi in providing environmental conditions of PWN. Chapter 3 clarified that fungal species and their distribution affected the density and distribution of PWN at the microhabitat level. The other study (Chapter I) has indicated that nematode community had little interrelation with PWN and that water content did not explain their population density. Since many other biotic and abiotic factors could affect the microhabitat conditions of pinewood (Shigo, 1967), further studies are needed to explore other factors that affect PWN population and distribution.

ABSTRACT

The pinewood nematode (PWN, *Bursaphelenchus xylophilus*), the causal agent of pine wilt disease, has association not only its vector beetle (*Monochamus alternatus*) and the host pine tree, but also with various organisms inhabiting the tree. The present study investigated the changes in nematode fauna and in microbial flora in 15-year-old Japanese black pine (*Pinus thunbergii*) trees over a year after inoculation with PWN in June 2004 and introduction with larvae of *M. alternatus* in July 2004. Fifteen species of nematodes were isolated from woody tissues of the pine trees; one species in each of Mononchida and Plectidae, two species in each of Monhysterida, Rhabditida and Tylenchida, three species of *Bursaphelenchus* including the PWN, and four species of Diplogasterida. The PWN and one species of Diplogasterida were the most prevalent nematodes isolated and these two species showed synchronized fluctuation in their populations. In addition, both PWN and the Diplogasterida species were more abundant around the pupal chambers of *M. alternatus* than elsewhere in the tree.

Each of the 15 nematode species obtained was characterized by microscopic observation and the bionomics and the general characters of each species were described especially in aspect of the feeding habit. Based on their morphological characters of oral aperture, *Bursaphelenchus* and Tylenchida were categorized as fungal feeder, and species of Diplogasterida, Monhysterida, Rhabditida and Plectidae as bacteria feeder or saprophagous species. The predatory Mononchida was much less in number than the others.

Fungal isolation was also conducted using the same pine trees inoculated with both PWN and *Monochamus* larvae, and eighteen species of fungi were obtained, including

Phialophora repens, *Sphaeropsis sapinea*, *Pestalotiopsis* spp., and *Rhizoctonia* sp., which were detected most frequently every season. The relationship between the PWN and fungi was examined using two indices, NPR (nematode population ratio) and ICA (index of cohabitation ability). The results showed that the dominant fungal species, except for *Rhizoctonia* sp., had a compatible relationship with PWN, where the PWN displayed a tendency to coexist with and/or propagate well on them, while *Rhizoctonia* sp. and *Penicillium* spp. had neutral, and *Trichoderma* spp. had an incompatible relationship with PWN.

To confirm the effect of fungal flora on the population density of PWNs, laboratory experiments were carried out using 18 species of fungi isolated from pine trees and *Botrytis cinerea* as control. All fungi, cultured on potato dextrose agar medium, served for PWN as food, and population growth of PWN on them was compared at 20°C. PWN propagated on *Pestalotiopsis* spp., *S. sapinea*, *P. repens* and *B. cinerea* from 10 to 15 days after inoculation, and the population on *S. sapinea* or *B. cinerea* was significantly higher than that on any other fungus. On *Rhizoctonia* sp. and minor fungi, PWN population was significantly smaller than that on dominant fungi. These results were consistent with those obtained in the field study.

Thus I conclude that some of the dominant fungi such as *S. sapinea*, *Pestalotiopsis* spp., and *P. repens* play important roles in determining the propagation and distribution of PWNs in dead pine trees.

LIST OF PUBLICATIONS

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